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3iRegeneration

Molecular Mechanism of Multipotent Cardiac Progenitor Cell Differentiation: Drug Discovery and Signaling Pathways

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Abstract

This body of research focuses on establishing a drug screening pipeline for discovering drugs which increase the differentiation of pluripotent stem cells into cardiac myocytes, known as cardiogenic molecules. Cardiomyocytes can be utilized in regenerative medicine by offering a platform for testing molecules or drugs which may increase cardiomyocyte proliferation and for using cardiomyocytes produced outside of the body for clinical transplant, in order to heal the damage caused by heart attacks. Building on known models and developmental pathways three assays were designed and implemented for *in vitro* cardiogenic molecule screening. A pipeline comprised of three primary screening systems; an embryoid body (EB) model, a cardiomyocyte directed differentiation model, and a magnetic activated cell sort (MACS) model. The MACS model uses the cell surface receptors Fetal Liver Kinase 1 (FLK1) and/or Platelet Derived Growth Factor Receptor alpha (PDGFRα) as the most practical platform for screening drugs against an enriched mesodermal population of cells. The MACS system was confirmed with flow cytometry to ensure the enrichment of Myl2-eGFP+ (ventricular cardiomyocytes) cells in the FLK1+ cells. Furthermore unique known molecules help elucidate the molecular mechanisms governing cardiomyocyte differentiation, measured by cardiomyocyte purity in *in vitro* models. Also demonstrated are assay controls which decrease purity and acts as negative controls for the MACS assay such as a late stage GSK-3 Inhibitor treatment used to constitutively activate the canonical Wnt/β-catenin pathway and effectively reduce the cardiomyocyte proliferation. Additionally, an early stage Wnt Inhibitor compound IWP-4 was used as a potential positive control effectively blocking late stage activation of canonical Wnt/β-catenin pathway and increase the *in vitro* purity of cardiomyocytes. These controls provide two important reference points for the many molecules screened over the course of these experiments for the 3i Regeneration project. Additional molecular inhibitors are used to elucidate the mechanism of action within the MACS cells; including a Sonic Hedgehog inhibitor (cyclopamine), an NKX2.5 activator (ISX-9) and a novel small molecule (C1). These models act as an effective pipeline bringing a potential drug through first an EB model, followed by a cardiomyocyte enriched model, to finally a MACS model targeting FLK1. This pipeline tests the molecules against conditions of increasing resemblance to the native microenvironment of a cardiomyocyte.

Key Words: cardiac progenitor cells, cardiogenesis, molecule screening, cardiomyocyte

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Abbreviations:

BMP4: Bone Morphogenetic Protein 4

BSA: Bovine Serum Albumin

DMSO: Dimethyl Sulfoxide

EBDM: Embryoid Body Differentiation Media

EB: Embryoid Body

eGFP: Enhanced Green Fluorescent Protein

ES Media: Embryonic Stem Cell Media

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FLK1: Fetal Liver Kinase 1

FGFb & 10: Fibroblast Growth Factor Basic & 10

HD: Hanging Drop

IMDM: Iscove's Modified Dulbecco's Media

LIF: Leukemia Inhibitory Factor

MACS: Magnetic Activated Cell Sorting

MEF Media: Mouse Embryonic Fibroblast Media

MTG: Mono-thio-glycerol

PBS: Phosphate Buffered Saline

PDGFRα: Platelet Derived Growth Factor Receptor Alpha

PFA: Paraformaldehyde

SFD: Serum Free Defined

2. Introduction and Literature Review

As the medical community seeks to improve the lives of patients afflicted by heart attacks it is important to first look at the current situation in the medical field and how the discovery of cardiogenic drugs and molecules for the treatment of damaged heart tissue comes to fruition. If a patient has a Myocardial Infarction (MI) the strength of their heart is greatly reduced by the death of cardiomyocytes which contribute to the muscle tissue in the heart. There are currently no medical treatments which address the repair or regeneration of these lost cells and no treatment which aims to return the damaged heart to its previous state. It is therefore of great interest to clinical medicine and researchers around the world to elucidate the mechanisms and developmental pathways of cardiomyocytes in order to produce new cardiomyocytes for patients. By examining the molecular mechanisms of cardiomyocytes and the *in vitro* models for their generation a complex model of developmental biology emerges. Building on the developmental pathways a new set of biological assay models for the screening of small molecules and drugs targeted for cardiomyocyte differentiation and proliferation can be built. The best way forward is to explore the molecule assays by design, and look at an assay design which allows screening of molecules against cardiac specific cell types.

In the event of a heart attack patients are given treatments which address the stresses on the heart and not the repair of the heart itself. Although diverse in nature, a heart attack most commonly results in a MI where the myocardium is cut off from the oxygenated blood flow it is afforded in a healthy human heart via the coronary arteries. It should be noted that within the context of a heart attack this thesis looks specifically at coronary heart disease and the impacts of loss of blood flow within the heart itself. Aspirin, nitroglycerin and oxygen therapies are immediately used on mild MI patients to work as vasodilators (nitroglycerin), aspirin (antithrombotic) and improve oxygen concentration (oxygen therapies). In more serious cases an intracoronary bypass can be used by excising a healthy vein from the body and surgically traversing the clotted coronary vein by adding a bypass vein. In addition to a bypass some patients may receive a percutaneous coronary intervention were a balloon stent is inserted directly into the clogged vein and expands to open the vein (NIH 2015).

Currently the medical community struggles to heal damaged hearts, in large part due to the hearts uniquely complex structure, and a lack of characterized resident cardiac progenitor cells (CPC). CPC are cells which are derived from the cardiac mesoderm and give rise to

cardiomyocytes, although it is still of considerably heavy debate whether resident cardiac progenitor cells even exist in the adult heart, the current consensus is that there is little intrinsic regenerative capacity in the adult heart. Along with the inability to engage in large scale repair of heart myocardium after an injury, the adult heart contains only limited long term regenerative potential, with the current consensus stating that there is approximately a 1% turnover in cardiomyocytes per annum throughout the adult life of the human heart (Mollova et al. 2013, Ali et al. 2014). This low turnover of the cardiomyocyte population is sufficient for a healthy individual, however in the face of large scale cardiomyocyte death, as in an MI, the heart struggles to regenerate.

2.1 Introduction to Heart Failure, A Look at Human Mortality and Morbidity

Many diseases plagued humans in the 19th century. Having revolutionized the treatment of patients in the early 20th century and having overcome many common disease models related to bacterial infection and hospital mortality, humans in the western world are living longer than ever. According to the National Institute of Aging (NIA) of the United States humanity's triumph over common infectious bacteria and the revolution in modern medicine rank as one of our species greatest achievements, with the average lifespan increasing drastically in the last decade and the record average set at 81 years old by Japan (Beard et al. 2011). With our longevity comes new disease models revolving largely around; the decay of cell systems, DNA maintenance, protein removal, and tissue dysfunction. The emergence of human pluripotent stem cells as an effective model for; developmental biology, regenerative modeling and pharmaceutical drug screening have opened the door for researchers to engage in novel lines of experimentation which seek to unravel the many molecular mechanisms found in adult human tissue systems (Hilcove et al. 2012, Zhu et al. 2013). Indeed one of our greatest challenges in advancing clinical medicine is to understand the developmental biology behind a functional tissue so it can be repaired or regenerated in times of injury or death. Pluripotent embryonic stem cells offer a unique model for deriving pluripotent cardiogenic stem cell populations for the repopulation of the heart and replacement of scar tissue (Rubart et al. 2006, Rubart et al. 2007, Field et al. 2007, Berlo et al. 2014). Although there are many disease models involving cellular decay in a diseased organ this thesis explores the disease of MI and its impacts on human mortality, morbidity and recovery.

2.2 The Disease of MI (Ischaemic Heart Failure)

MI is defined as the moment that the heart's blood flow rate drops below the needed level proportionate to the body's need for oxygen. With blockage in the heart valves the intra-chamber pressure increases immediately and causes failure in the ejection fraction and rhythmic flow of blood throughout the body, known as cardiogenic pulmonary edema (Szema et al. 2015). According to the World Heart Federation approximately 17,327,000 people die every year from heart failure, based on a study by the World Health Organization (WHO 2008). In addition 22.5% of these deaths globally will be attributed to ischaemic heart failure, when the coronary arteries supplying blood to the heart myocardium become blocked and result in the death of cardiac muscle tissue, a.k.a. MI.

MI is the end result of an ischaemic heart failure where blood flow to the heart via the coronary arteries has been reduced, disrupted, or blocked, causing a loss of oxygenated blood to the resident cells. The lack of blood flow through the coronary arteries is caused by a buildup of oily lipids and proteins known as plaque. This buildup of plaque is called atherosclerosis and occurs over a lifetime, and is dependent on a number of lifestyle choices such as; the physical fitness of an individual, dietary choices, and genetic profile. The reduced flow of oxygenated blood and clogging of a coronary artery, called coronary heart disease (CHD), causes an inadequate supply of blood and thus an inadequate oxygen supply to vital heart muscle cells, aka cardiomyocytes. As the clog persists oxygen starvation causes cells in the periphery of the heart, most commonly the left ventricle, to begin to die. The mass die off of cardiomyocytes as a result of CHD is called a MI and results in the loss of approximately one billion resident cardiomyocytes from the functional heart. Symptoms of MI include; chest pain or discomfort, upper body pain or discomfort and shortness of breath. More intense MI can lead to collapsing, fainting and too commonly death. A majority of MI deaths occur within the first hour after the onset of symptoms (Horburger et al. 2014, NIH 2015). Although the initial MI causes a majority of deaths survivors are left with a damaged heart. Damage for survivors occurs over the next several days as the heart repairs the damaged muscle by having cardiac fibroblasts; weave a network of scar tissue into the damaged area and remove the necrotic cells (Jazwinska et al. 2015). Unlike other diseased tissues in the adult human, such as liver and skin, the heart is incapable of massive cellular repair. Categorized as non-regenerative healing the replacement

of necrotic tissue with scar tissue blocks the restoration of the heart muscle and although relieving damage to the architecture of the heart it ultimately slows and disrupts the heart's function (Jazwinski et al. 2015). This scarring may save the damaged tissue but it poses a serious problem for the future health of the adult heart often causing more disruption in the heart function, to the point where an MI patient may have trouble walking to the bathroom or getting out of bed.

2.3 Cardiogenesis and the Formation of the Chambered Heart

In order to best characterize cardiomyocytes for applications in regenerative medicine a full understanding of the origins of cardiomyocytes and cardiogenic mesoderm must be obtained, from a developmental perspective. Of the three developmental cell lineages; mesoderm, ectoderm and endoderm, a mature heart is derived almost exclusively from the mesodermal cell lineage. Immediately after the three germ layers form, the mesoderm migrates between the ectoderm and endoderm, with respect to the amnion and yolk sac. This motion is followed by the lateral extension of the mesoderm and cranial expansion along the embryonic disc. This early organization allows the proliferation of mesoderm to produce an axial structure which gives rise to the lateral plate mesoderm (Moorman et. al. 2003). All the mature cells of the adult heart originate from two embryonic cell populations called the Primary Heart Field (PHF) (also called First Heart Field) and the Secondary Heart Field (SHF), which make up the Cardiac Crescent (Figure 1). The heart is the first fetal organ to function and it first functions in mouse models on E8.0 (Embryonic day 8.0), and E20 in Humans, where the PHF and SHF expand in population and the SHF migrates to the hearts midline forming a linear heart tube with non-specific chamber-like regions. These two dense cell regions first become visible around E7.0 of gastrulation in the mouse model, or week three in humans. It is important to note here that during early development there are a number of genes which are sequentially activated and inactivated which guide the cell fates of the progeny of the Cardiac Crescent. Specifically genes for NKX2.5 and Gata4 (Groot et al. 2005) mark the earliest stages of heart development.

The Heart Tube is formed when the midline of the PHF and the SHF fuse. This fusion is the cornerstone of tube formation which leads to the contraction and flow of fluids in the developing embryo. More importantly the fusion sets the stage for a spatial arrangement of the

developing Heart Tube where the expansion of populations along the tube, called ballooning, allows for looping of the Heart Tube and establishment of an anterior specific population (ventricular) and posterior specific population (atrial).

2.4 Looping of the Heart Tube and Chamber Formation

At E8.5 in mouse models the linear Heart Tube undergoes a rightward looping motion causing the formation of a distinct left-right orientation and distinct cell populations contributing to non-septated chambers. This looping of the heart is driven by uneven growth and as a result forms two uneven primitive chambers dubbed the primitive ventricle and primitive atria. It should be impressively noted that the organ is functioning, albeit at a limited capacity, throughout remodeling and development, providing essential fluid exchange and oxygen transport to all the organ systems of the developing embryo. By E10.5 in mouse models the looping and uneven growth of the heart has caused the venous poles to shift anteriorly creating a physical layout which sets the heart up for proper development of the septated chambers (Bassel-Duby et al. 2013). The E10.5 is termed Chamber Formation (Figure 1) and results in the septation of the chambers via flap like muscle tissue which act as a one way valve to drive the blood flow in the correct direction.

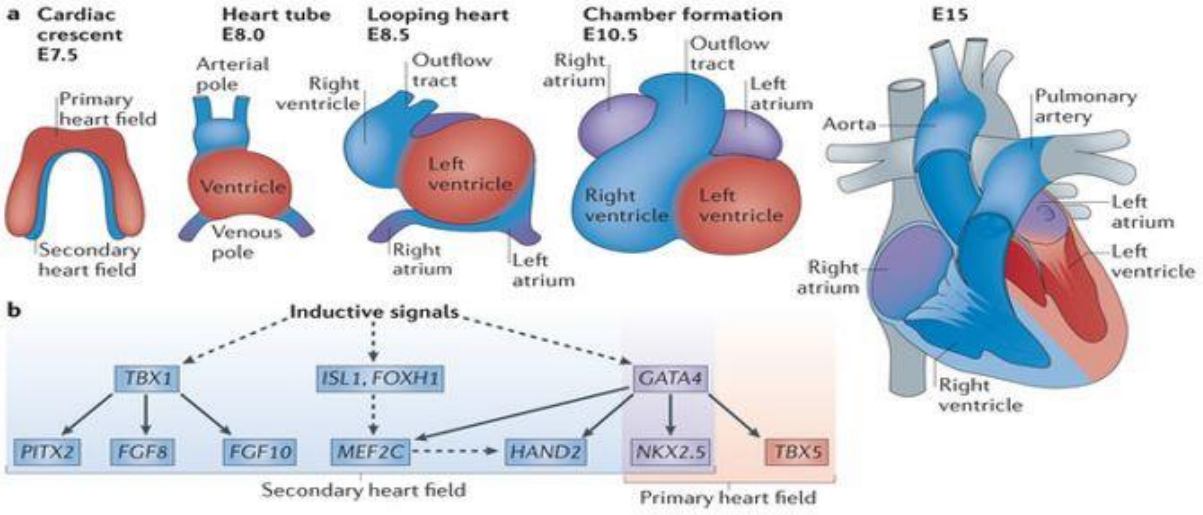

Figure 1: Developmental stages of the mouse heart. The mouse heart first functions as a beating immature organ at E8.0 and within *in vitro* assays beating cardiomyocytes are visible Day 7-8. The methodical development of the heart is shown and each unique structure is dependent on the activation of specific molecular mechanisms which drive the proliferation of specialized cell populations (Xin et al. 2013).

2.5 The Adult Heart

Development of the human heart is a complex event involving several weeks of fetal gestation and yields one of the most complex organs in the adult human body. Unlike other organs in the human body the heart begins to beat around E8.0 in mouse and E18-E21 in humans, and continuously beats until the end of life. Without a single rest the unending beating of the heart supplies all the oxygenated and deoxygenated blood, plasma and blood borne nutrients for every organ in the total organ system of the human body. The average human body has approximately 4.7 liters of circulating blood to provide the body with all its gas exchange needs (Cameron et al. 1999). The adult heart contains; 4 chambers, 2 vena cava (carrying deoxygenated blood into the heart), 2 pulmonary arteries (carrying deoxygenated blood to the lungs via the pulmonary trunk), 2 pulmonary veins (carrying oxygenated blood from the lungs into the heart), and 1 aorta (carrying oxygenated blood to the spinal cord and down the spinal column (Iaizzo et al. 2009).

2.6 Biology of Resident Heart Cells

Within the developing heart, embryonic cells traverse a carefully choreographed Waddington's landscape where the spatial and temporal expression of cardiac gene cassettes drives cells to their specialized fates. Although our understanding of the genetic profile of cardiomyocytes is always evolving, the current consensus is that major gene regulatory networks control cardiomyocyte differentiation such as; Gata, Hand, Islet, Mef2, Mesp, and Nkx (Bruneau et al. 2010). Currently there are two known cell lineages which contribute to the adult heart. These lineages are derived from the mesoderm and ectoderm germ layers during embryogenesis. Within these two germ layers there is some debate as to the current consensus on the total number of cell types in the adult heart. From the mesodermal layer you can define the Brachury+ mesodermal lineage as a first marker of functional mesoderm on E3.0 (Später et al. 2014). Following a differentiation landscape cells can further be classified into cardiac mesoderm population on E4.25 which is currently defined as Fetal Liver Kinase 1+ (FLK1+), Mesodermal posterior 1+ (Mesp1+), Platelet Derived Growth Factor Receptor alpha+ (PDGFRα+), Stem cells antigen 1+ (Sca1+), tyrosine-protein kinase Kit+ (C-kit)+ and Insulin gene enhancer protein 1+ (Isl1+) (Kattman et al. 2006, Martin-Puig et al. 2008, Chien et al. 2008, Später et al. 2014, Chong et al. 2014). All cells are derived from the mesodermal lineage

and all cells are derived from a common pool of Primordial Cardiovascular Progenitors from the FHF and SHF. These multipotent cardiac progenitor cells give rise to the specific lineages of the functional heart cells defined by their genetic profile. The unique cell types which populate the heart are further identified by their individual gene expression profiles and cell surface receptors (Figure 2).

Cells Type:	**Known Markers:**
Smooth Muscle	SM-MHC+
	SMA+
Right Ventricular Cardiomyocytes	Mlc-2-v+
	cTnT+
Atrioventricular Nodal Cardiomyocytes	HCN4+
Atrial Cardiomyocytes	SLN+
	cTnT+
	Mlc-2-a+
Sinoatrial Nodal Cardiomyocytes	HCN4+
Endothelial Cells	CD31+
	vWI+
Vascular Smooth Muscle Cells	SM-MHC+
	SMA+
Smooth Muscle Cells	SM-MHC+
	SMA+
Atrial and Left Ventricle Cardiomyocytes	NKX2.5+
	Mlc2a/v+
	cTnT+

Figure 2: Known active genes used to characterize cell types. The ubiquitous expression of cTnT in all cardiomyocyte cell subtypes offers a reliable quantifiable tool for identifying cardiomyocytes. Additionally it can be seen that all ventricle cells have Mlc2a/v which is also called Myl2, our reporter line utilized in these experiments. (Martin-Puig et al. 2008, Später et al. 2014)

2.7 Molecular Mechanisms of Mesodermal Specific Cardiogenic Cell Lineage

The specification of pluripotent stem cells to mesoderm requires the induction of four unique molecular pathways; Activin/Nodal, Bone Morphogenetic Protein (BMP), Wnt, and Fibroblast Growth Factor (FGF) (Kimelman et al. 2006) (Figure 3). The current consensus is that the most applicable way to generate cardiomyocytes *in vitro* is the temporal activation of canonical Wnt/β-catenin pathway through Activin/Nodal via Activin A and BMP4 cytokines,

which leads to the intracellular activation of β-catenin and the induction of mesodermal FLK1+ cell lineages (Nostro et al. 2007, Lian et al. 2013). In addition, the generation of cardiomyocytes requires the activation of the SMADs pathways via BMP4 (Anderson et al. 2008). Both Activin A and BMP4 belong to the Transformation Growth Factor β (TGF) superfamily and these cytokines work through binding to Type I and Type II cell surface receptors and activate the SMAD signal cascade to induce intracellular transcriptional activation of target genes (Chen et al. 2004, Varga et al. 2005, Xia et al. 2009). SMADs are the primary signal transducers for the intracellular gene activation of the TGF-β superfamily of receptors and are activated through the serine/threonine kinase receptors Type I and Type II (Miyazono et al. 2005). The crosstalk between the Wnt/β-catenin and BMP/SMAD pathways is the key component in deriving cardiac specific mesoderm (Qi et al. 2004, Caliceti et al. 2014, Kim et al. 2015, Jain et al. 2015). With the activation of these pathways and induction of mesoderm it then becomes necessary to activate the Fibroblast growth factor receptor (Fgfr) mediated Mitogen-Activated Protein Kinase (MAPK) pathway via Fibroblast Growth Factors Basic and 10 (FGFb and FGF10) (Kimelman et al. 2006). All these pathways are activated by the TGF-β superfamily of cell surface receptors and are activated primarily by BMP4, Activin A, FGFb and FGF10 in our experiments. When presented as linear signal transduction pathways it should be noted that there is in fact a great deal of crosstalk between these molecular mechanism, particularly Nodal and BMP which utilize similar SMAD signal transduction pathways

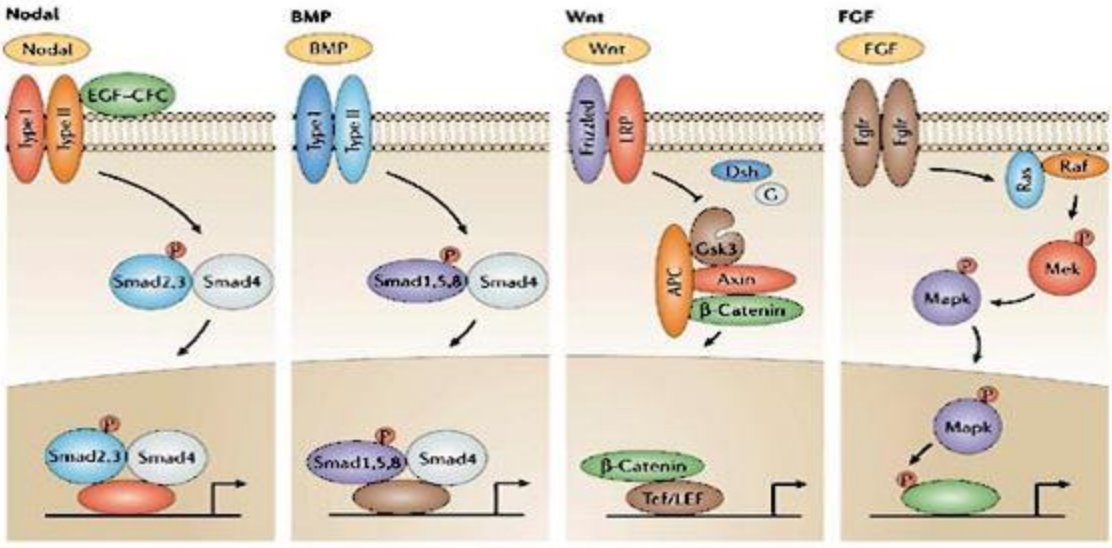

Figure 3: Active molecular mechanisms during mesodermal induction. This diagram of the signaling pathways presents the canonical depictions of the required pathways for the induction of the mesodermal cell lineage (Kimelman et al. 2006).

2.8 Cardiac Stem Cell Therapies for Heart Disease

The generation of new heart muscle through the differentiation and replacement of lost cardiomyocytes in an MI patient requires the generation of new cardiomyocytes *in vitro*. From a clinical perspective generating these cells could be used in constructing a cell patch or transplantable population of cells, which could be implanted into diseased or dead cardiac tissue and serve as a replacement to the cells lost during a MI. This is known as repair and involves the generation of mature, functional and differentiated cells to be transplanted on a scaffold into the damaged tissue. The use of human derived cardiomyocyte in a cell transplant in a rat model after an MI has been shown to improve the function of the rat heart (Laflamme et al. 2007). In fact Laflamme et al. showed that along with the survival of cardiac specific cell types in the transplant, noncardiac cell types died and were harmlessly flushed away from the heart. Simultaneously they showed thickening of the infarcted wall, indicating that the transplanted cells assimilated with the native cardiomyocytes and contributed to the systolic force, improving the ejection fraction of the infarcted heart. Although their experiment had a small sample size there have been successful transplants of Embryonic Stem Cell (ESC) derived cardiomyocytes into humans using microscale cardiospheres, small bodies of cells containing cardiomyocytes and CPC. One unique study currently in phase 1 clinical trials utilizes cardiosphere-derived cells (CDCs) by implanting autologous intracoronary CDCs into patients

afflicted with MI in the hopes of assessing the safety and efficiency of cardiosphere transplants (Makkar et al. 2012). Although this study is preliminary it highlights the turning point reached in the applications of clinical medicine. As our understanding of the mechanisms of cardiomyocytes expands we are edging closer and closer to a clinically relevant treatment for MI patients using ESC derived cardiomyocytes.

The assimilation and maturation of cardiomyocytes is one of the most pressing issues in defining a successful cell transplant to the damaged heart. Current methods have utilized a combination of electrophysiological stimulation and contractile function to mature cardiomyocytes *in vitro* (Blazeski et al. 2012, Parsa et al. 2015). In fact Opie et al. showed that cardiomyocyte maturation is highly dependent on mechanical loading, the amount of electrical stimulation driving their contractility (Opie et al. 2006). In addition to mechanotransduction, the mechanical forces acting on the cell within its environment, the maturation of cardiomyocytes has been shown through chemical means using Tri-iodo-L-thyronine, aka Thyroid hormone (Chattergoon et al. 2007, Yang et al. 2014). These studies offer interesting insights into the nature of cardiomyocytes during transplantation. On the one hand it is most optimal to have fully functional cardiomyocytes for transplant, to prevent uncontrolled proliferation and tumor formation. However there is also a benefit from having immature cardiomyocytes in a transplant that will proliferate and fill the empty space left by cell death from MI. The optimal target would be to have a drug based system to temporally activate the molecular mechanisms which drives the proliferation of only cardiomyocytes. Work by Keating et al. shows that cardiomyocyte proliferation can be induced using early stage treatment with a GKS-3 inhibitor compound which plays a key role in the intranuclear regulation of the Wnt/β-catenin pathway (Tseng et al. 2006). In addition to a GSK-3 Inhibitor it has been shown by Gianakopoulos et al. that the cross talk between BMP and a Sonic Hedgehog inhibitor (SHH) can also activate cardiomyogenesis in P19 cancer cells (Gianakopoulos et al. 2009). The discovery and *in vitro* study of molecular inhibitors is of great value to the advancement of our knowledge in controlling the cell cycle of cardiomyocytes. The maturation of cardiomyocytes for cell transplant in patients remains an area of great interest and in need of further study.

2.9 FLK1 and PDGFRα Cell Surface Markers for Mesodermal Cardiac Progenitor Cells

There is mounting evidence that FLK1, also called Kinase Insert Domain (KDR), Vascular Endothelial Growth Factor Receptor 1 (VEGFR1), or Cluster of Differentiation 309 (CD309) and PDGFRα, or Cluster of Differentiation 144 alpha (CD144α), are positive selection markers for the *in vitro* selection of multipotent cardiac progenitor cells from heterogeneous ESC cultures (Yamashita et al. 2000, Kattman et al. 2006, Martin-Puig et al. 2008, Kattman et al. 2011, Lian et al. 2013, He et al. 2014). The Keller lab also showed that FLK1 marks hematopoietic progenitor cells on D3.25 and multipotent cardiac progenitor cells on D4.25 (Kattman et al. 2011). When cell populations are positively selected through antibody mediated Magnetically Activated Cell Sorting (MACS) cardiomyocyte purity of >50% in *in vitro* models have been reported (Kattman et al. 2006, Kattman et al. 2011, Hartman et al. 2014).

2.10 Pharmacology and Pharmacotherapy Techniques for Drug Screening

It is safe to say that in the current healthcare environment every major pharmaceutical company is engaged in high-throughput drug screening. Although many assays exist for testing; drugs, synthetic compounds, cell derived extracts, modified small molecules and novel compounds they all must ultimately be screened against a biological system which resembles their targeted biological environment. For this purpose the Embryoid Body (EB) system is a likely candidate for *in vitro* drug screening. The automation of high-throughput EB drug screening models offers high accuracy and short experimental assay time (Kowalski et al. 2012). In addition to the scalable nature of EB generation, the 3 dimensional (3D) nature of an EB offers a physiologically relevant model for drug discovery and drug screening (Devalla et al. 2015). The advantages of EB drug screening are that you can contain within a single 3D system a heterogeneous cell population reminiscent of the cells found in the developing embryo (Ader et al. 2014, Pettinato et al. 2015). An EB is formed by gravity well, where a fixed population of pluripotent embryonic stem cells, 500 cells, is placed in a small liquid droplet, 20 µL of Embryoid Body Differentiation Media (EBDM) (Figure 4), and grown upside down in a V-bottom plate. This causes a half circle for the cells to settle in. As they come together they form an aggregate or sphere. Within the EB there develops a spatial orientation which drives germ layer formation.

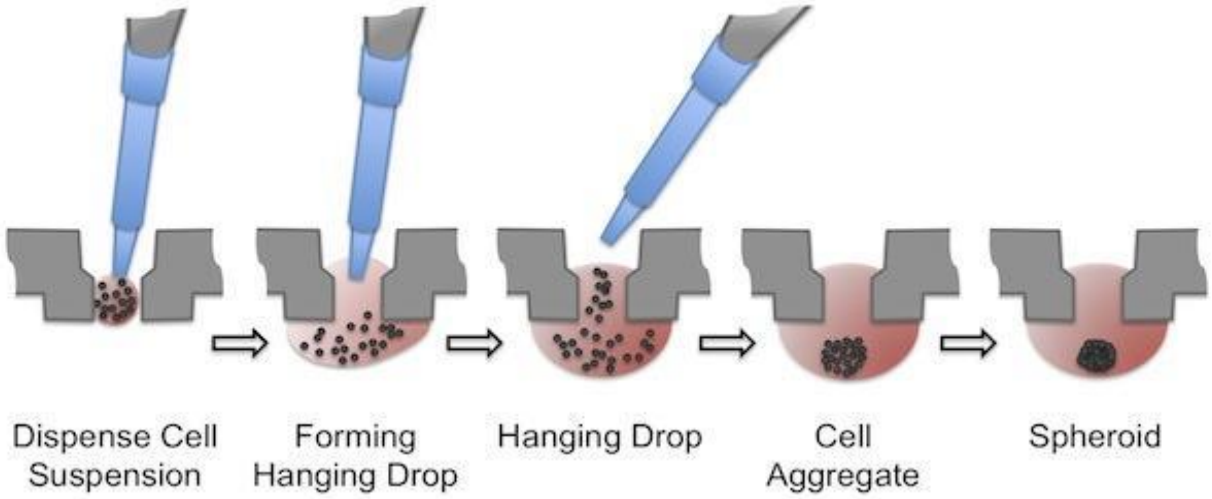

Figure 4: Formation of an EB via hanging drop, aka. gravity well (Figure from 3D Biomatrix). The EB formation depicted here is effective at producing a single EB of uniform size and shape per media droplet. (http://www.cellsystemsbiology.com/products_3dbiomatrix.php)

2.11 Establishment of a 3D EB Based Molecule Assay

To determine the effects of a drug or small molecule on a population of cells a heterogeneous (mixed) population of cells can be generated through a spontaneously differentiated EB. This *in vitro* drug screen has many advantages and a few key disadvantages. As the cells proliferate they form a spatial construct which yields the three germ layers mimicking the cell populations found in an embryo, albeit without the specialized organization. The advantages of this EB molecule assay are that drugs can be tested against a diverse population of cells and the toxicity of the drugs can be checked in a short 5 day experiment in order to optimize the drug concentration for more precise experiments. The drawbacks of this assay is that with such a diverse cell population it is challenging to identify; the specific stoichiometry of the drug and how it interacts with the cell's glycocalyx, the crosstalk between cells interacting in a small microenvironment, and the effects of the molecule on one cell type versus another cell type. In addition, the 3D structure of the spontaneously differentiating EB brings into question the penetrance of the drug or molecule as the EB lacks proper vasculature for fluid exchange and thus the drug or molecule must rely solely on diffusion to enter the EB. Finally, the largest problem with using a diverse cell population to screen for cardiogenic molecules is an inherent feature of cardiomyocytes, they proliferate slowly when compared to other cell types. *In vitro* assays which generate cardiomyocytes are limited to Day 12 or Day 16 of differentiation in 3D EB models because the cardiomyocytes will become overgrown by

other cell types. In fact the differentiation of cell types in the EB will eventually lead to blood islands and microvasculature which acts to move fluids throughout the EB. This late stage EB growth however was not used in these experiments. The EB model has also been shown to generate ventricular cardiomyocytes (Muller et al. 2000) and atrial cardiomyocytes (Devalla et al. 2015) making it an all-encompassing model system for molecule screening targeted for the generation of ventricular and atrial cardiomyocytes.

2.12 Establishment of a 2D Monolayer Based Molecule Assay

In search for cardiogenic drugs and molecules which will induce cardiomyocyte proliferation it becomes apparent that the spontaneous differentiation model falls short. In order to screen drugs against a more homogenous population a directed differentiation model can be used on a monolayer. In this model BMP4, Activin A, Vascular Endothelial Growth Factor (VEGF) and FGFb & 10 can be used to produce a mesodermal population which has an enriched cardiomyocyte purity compared to the EB model (Kattman et al. 2006, Kattman et al. 2011). The clear advantages of this model are that a defined population of cells. The disadvantages of this model are that the use of cytokines already activates procardial gene cassettes and thus the molecule's activity may be overshadowed. However, the 2D monolayer is practical for low throughput drug screening where there is a high probability of a cardiogenic molecule. Testing a compound in the 3D spontaneously differentiated EB in order to test basic toxicity and cardiogenic effects on a population can work as an introductory screen to eliminate unsuccessful molecules. Drugs or molecules which pass the 3D molecule assay could then be tested in the 2D directed differentiated model. It should be noted that the monolayer is not a true 2D model as multiple cell layers form, however compared to the spatial arrangement of the 3D EB it offers a significantly different drug testing platform.

2.13 Establishment of a Mesodermal Cardiogenic Molecule Assay

The establishment of a molecule screening assay using both the 3D spontaneous differentiation model and the 2D directed differentiated model both have advantages and disadvantages with the problem of mixed cell populations and unknown cell types being the largest issue. In addition to unknown cell types interfering with the assay, there is an emerging emphasis on the value of a 3D model over a 2D model as cells in the native heart never interact

with a 2 dimensional like space and thus clinical research benefits more from 3D modeling (Kurokawa et al. 2015). Using specific cell-surface antigens one can sort the cells using MACS which employed magnetic conjugated antibodies for surface markers which mark multipotent cardiac progenitor cells. Using the FLK1 and the PDGFRα cell surface antigens one can sort the cells for a more defined population.

2.14 Myl2-eGFP Reporter Line and Cardiac Troponin T

To quantify ventricular cardiomyocytes through fluorescent imaging and flow cytometry a mouse embryonic derived E14 reporter line for Myl2-eGFP cells was used. The myosin light chain 2 (Myl2) gene encodes a sarcomeric protein that is a member of the EF-hand calcium binding protein superfamily and encodes sarcomere protein which contributes to functional striated muscle (Sheikh et al. 2015). The functional sarcomere protein derived from the Myl2 gene is specific to ventricular cardiomyocytes and indicative of a functional ventricular cardiomyocyte (Bizy et al. 2013). For these reasons Myl2 offers an excellent source for the detection of ventricular cardiomyocytes *in vitro*.

In addition to the use of a reporter line for ventricular cardiomyocytes, Cardiac Troponin T (cTnT) immunocytochemistry can be used to label both ventricular cardiomyocytes and atrial cardiomyocytes for flow cytometry. CTnT is a regulatory protein that is specific to myocardium and has been shown to be pancardial, occurring in all cardiomyocytes (Sharma et al. 2004). For this reason cTnT offers an excellent labeling mechanism for positively identifying myocardial derived cardiomyocytes in *in vitro* modelling.

3. Research Objectives

The goal of this research was to develop a cardiogenic molecule assay utilizing known developmental pathways and screen novel compounds. Here is outlined three molecule assay models which individually offer unique perspectives on the mechanistic actions of known drugs and novel compounds. Building on previous works in cell culturing techniques and the molecular mechanisms of cardiomyocyte differentiation this thesis work seeks to establish a consistent, accurate and reproducible set of cardiogenic molecule assays which allow the development of a drug screening pipeline from an EB model, to a cardiomyocyte enriched model, to a specialized mesoderm molecule assay.

4. Material and Methods

4.1 Media Index:

1. Dulbecco´s Modified Eagle´s Medium (DMEM) with 4,5 g/L Glucose, without L-Glutamine (Cat # BE12-614F) (Lot # 5MB059), Bio Whittaker, Lonza.
2. Iscove´s Modified Dulbecco´s Medium (IMDM) with NaHCO3, without L-Glutamine (Cat # I3390) (Lot # RNBD5178), Sigma Life Science, Sigma-Aldrich.
3. Ham´s F-12, 1X (Modified) with L-glutamine (Cat # 10-080-CVR) (Lot # 10080145R), Mediatech Inc., Corning.
4. Embryomax Ultrapure Water with 0.1% Gelatin (Cat # ES006-B) (Lot # 50129-1 L4), Specialty Media, Millipore.
5. Fetal Bovine Serum (FBS), origin: South America (EU approved origin) (Cat # 10270-106) (Lot # 41G7530K), Gibco, Life Technologies.
6. StemPro-34 SFM (1X), SFM for Hematopoietic Stem Cells, without L-glutamine, use with StemPro34 nutrient supplement (Cat # 10640-019) (Lot # 1685362), Gibco, Life Technologies.
7. Phosphate Buffered Saline (PBS) without Ca and Mg (Cat # BE17-516F) (Lot # 5MB011), Bio Whittaker, Lonza.
8. Minimum Essential Medium Nonessential Amino Acids (MEM NEAA) (100X) (Cat # 11140-035), Gibco, Life Technologies.
9. 2-Mercaptoethanol, (1000X) (Cat # 21985-023) (Lot # 1517482), Gibco, Life Technologies.
10. ESGRO mLIF Medium Supplement (Cat # ESG1107) (Lot # 2297438), Millipore.
11. GlutaMAX (100X) (Cat # 35050-038), Gibco, Life Technologies
12. 1-Thioglycerol, liquid bioreagent, suitable for cell culture, (Cat# 1001789542) (Lot#MKBR3425V), Sigma Life Sciences
13. Penicillin Streptomycin (Cat# 15140-122) (Lot# 1677650), Gibco by Life Technologies
14. Mycoplasma-ExS Spray (Cat# PK-CC91-5051) (Lot# 395P035), PromoKine

Materials
1. CellStar 96 Well Cell Culture Plate, sterile, F-bottom, with lid (Cat # 655-180) (Lot # E150435E), Greiner bio-one
2. Cellstar 12 Well Cell Culture Plate, sterile, with lid (Cat # 665-180) (Lot # E150635X), Greiner bio-one.
3. Cell Strainer, 40 µm nylon (Cat # 352340), Falcon, A Corning Brand
4. MS magnetic separating column, (Cat # 120-000-472) (Lot # 5150223010), MACS Miltenyi Biotec
5. OctoMACS Separator Magnet (Cat# 130-042-109) (Lot# 5140527430), Miltenyi Biotechnology
6. MACS MultiStand, Miltenyi Biotechnology
7. Minisart Single use filter unit, (Cat# 16532) (Lot# 16532 21327103), Sartorius stedim

Growth Factors/Cytokines
1. rhVEGF, Vascular Endothelial Growth Factor, recombinant human (SF21-derived) (Cat# 293-VE) (Lot# 115015051), >97% purity, 10 µg, R&D Systems
2. rh/m/r Activin A, recombinant human/mouse/rat (CHO cell-derived) (Cat# 338-AC) (Lot# BNV3615041), >95% purity, 10 µg, R&D Systems

3. rhFGF-10, recombinant human Fibroblast Growth Factor 10 (*E. coli*) (Cat# 345-FG) (Lot# ASP-2914091), >97% purity, 25 µg, R&D Systems
4. rhBMP4, recombinant human Bone Morphogenetic Protein 4, (NSO-derived) (Cat# 314-BP) (Lot# BEM 9514081), >95% purity, 10 µg, R&D Systems
5. rhFGFb, recombinant human Fibroblast Growth Factor Basic, (*E.coli*) (Cat# 233-FB) (Lot# HKW11714121), >97% purity, 25 µg, R&D Systems

Antibodies and Staining Tools
cTnT, Troponin T Ab-1, (13-11), (ref# ms-295-p1) (Lot# 295P1402F), 0.5 ml, 0.2mg/ml, Lab Vision Corporation, Thermo Scientific.
647 Bovine anti-mouse, IgG-CFL 647, (cat# sc-362286) (lot# B0312), 200µg/0.5ml, Santa Cruz Biotechnology.
Vectashield Mounting Medium for Fluorescence with DAPI (Cat# H-1200), Vector Laboratories Inc.

Commercial Molecules
NKX2.5 activator: ISX-9, N-cyclopropyl-5-(2-thienyl)-3-isoxazolecarboxamide, Cat # 16165, Cayman: Chemicals
Sonic Hedgehog Inhibitor (SHH In): Cyclopamine, 11-deoxojervine, cat # C4116_Sigma, Sigma-Aldrich
Glycogen synthase kinase 3 Inhibitor (GSK3 In): CHIR 99021, cat # 4423, Tocris Bioscience
Wnt Inhibitor: Stemolecule Wnt Inhibitor IWP-4, Cat # 04-0036, STEMGENT

Methods

All experiments pertaining to the completion of this body of research were performed at; the Institute of Biotechnology, Helsinki, the Faculty of Pharmacy, University of Helsinki, and the FACS Core Unit in Helsinki Central Hospital´s Biomedicum, Helsinki. Work was supported by the 3i Regeneration group, TEKES, the Finnish Heart Foundation, and Biocentrum Helsinki.

4.2 Maintaining and Culturing Stem Cells

All experiments done in this body of research utilized mouse embryonic stem cells (mESCs) from an E14 line. The experiments used a reporter cell line for Myl2-eGFP for ventricular cardiomyocytes. In addition to the reporter line a limited number of experiments were performed utilizing a wild type E14 mouse cell line.

4.3 Storage and Initiating Cell Culture

MESCs are stored at -160℃ in cryovials and liquid nitrogen continuously. The cryovials were thawed in a 37℃ water bath for 4 minutes at which point the contents of the

cryovial; 1 ml of Freezing media and 1 ml of cells in Embryonic Stem cell media (ES Media), are washed in 8 ml of Mouse Embryonic Fibroblast media (MEF media, containing DMSO + 10 % FBS). All media used contains penicillin and streptomycin as an added measure to suppress potential bacteria growth. Thawed cells are then plated to a T25 gelatinized filter cap flask containing 6 - 8 ml of ES media. Filter caps must be used to ensure gas exchange between the T25 culture flask and the controlled environment of the incubator. ES media contains Leukemia Inhibitory Factor (LIF) which is the key ingredient in maintaining cells in the pluripotent state. Plated cells were incubated at 37℃, 5% CO2, and 90% humidity. For experimental use the cells were grown for 4 days post thaw to ensure pluripotency and confluency of the cell population. Thawing cells must be done in the afternoon (Day -4) and media changed to fresh ES media early the next day (Day -3) to reduce cell stress brought about by exposure to Dimethyl Sulfoxide (DMSO), a key ingredient in the freezing media. It may also be necessary to wash the cells with Phosphate Buffered Saline (PBS) during the first media change to remove dead cells and debris. As dead cells release necrotic factors which could influence the pluripotency of the vegetative cells.

4.4 Maintaining Cell Population and Splitting Cells

For all experiments the cells were split on Day -2, in order to control the cell population density and ensure the correct number of cells are ready for experimental use on Day 0. Splitting cells is a core component of correct maintenance of pluripotency within a cell population *in vitro*. The cells will form small colonies of pluripotent cells by Day -2 and are subsequently split (divided to a new growth surface) to prevent these cells from growing to a point where they make physical contact with their neighboring cell colonies. When the pluripotent cells make contact with other groups of cells they begin to flatten, elongate, differentiate and lose their pluripotency.

On Day -2 the media from the T25 flask was aspirated, and washed with 4-6 mls of PBS. After aspirating the PBS, 1 ml of 37℃ TrypLE Express 1x (Trypsin) was added to the cells and placed back in the incubator for 4 minutes. Trypsin is optimally activated at 37℃ and is an enzymatic agent used to dissociate cells from colonies and into a single cell suspension. The trypsin is then inactivated using 1 ml of 37℃ MEF media (inactivation via 10 % FBS). In order to ensure a single cell suspension, imperative to maintaining pluripotency of cells post-

split, the trypsin and MEF media (2 ml total) were pipetted over the cells in the flask via a high angle wash 10-15 times gently. After the cells have been washed they were transferred to a 15 ml tube, containing 8 ml of 37℃ MEF media. With the addition of the trypsin and MEF, cells were transferred to a new tube and gently pipetted to mix the total mixture 2-3 times to ensure total inactivation of the trypsin. It should be noted that trypsin is toxic to the cells and will kill the cells if exposure time exceeds a time limit (≅30 minutes). After inactivating the trypsin mixture was centrifuged to pellet the cells. The cells are resuspended in 1 ml of ES media and distributed to a new gelatinized T25 flask containing 6-8 ml of ES media, at 37℃. The distribution of cells is directly dependent on the density of the cell population before splitting. For a correctly maintained T25 flask the split should be about 1/10, for starting culture of 350,000 cells per T25 flask. Flasks should be stored in the incubator at 37 ℃, 5 % CO2 and 90 % humidity.

4.5 Initiating Experiments on Day Zero

For all experiments the media was changed on Day -1 to ensure the continued presence of viable LIF in the media and the pluripotency of the cells. On Day 0 the cells are ready for experimental use. The cells are trypsinized using the same procedure as Day -2. Instead of plating the cells, they are left in the 15 ml tube and washed two times using IMDM, centrifuged and the media aspirated between each wash. The cells were resuspended in 1 ml of Serum Free Defined media (SFD containing MTG and Ascorbic acid) for a directed differentiation experiment or a mesodermal molecule assay. For a hanging drop molecule assay the cells are resuspended in EBDM and proceed to section 4.6. Note that at this point the cells are no longer treated with LIF and thus they will begin to spontaneously differentiate.

4.6 Hanging Drop for EB Drug Screen

For a hanging drop molecule assay you must have 500 cells per 20 µL of EBDM for each well of a 96 well V-bottom plate. The cells are pipetted into each well and tapped down into the plate on the table to ensure the droplets have sunk into the bottom of the V-bottom well. The inverted plates are incubated for 2 days. On Day 2 the plates are turned over to its correct orientation and molecules added to each well, diluted into EBDM 1:1000, bringing the total volume of the well up to 200 µL. Molecules are then present in media and incubated for 3 days,

this is the first exposure D2-D4 (Figure 4). On Day 2 there will be an observable EB inside each well, with approximately 10% of wells not forming EBs. On Day 5 the EBs are transferred to a 12 well gelatinized cell culture plate containing EBDM. On Day 6 the media was aspirated and new EBDM with molecules was added. After 4 more days media was changed to molecule free EBDM media and the cells are trypsinized on Day 12 for flow cytometry of cTnT and Myl2-eGFP.

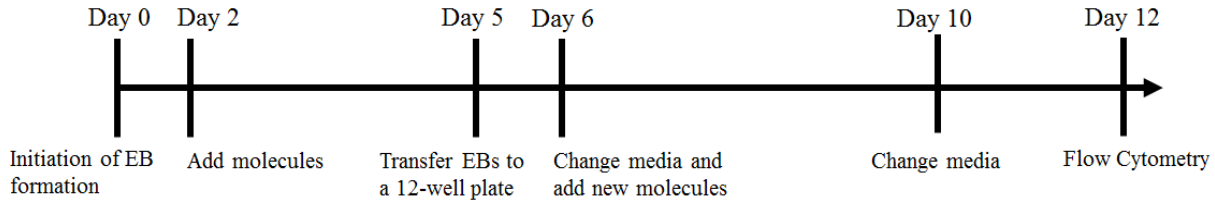

Figure 5: Timeline for EB formation and small molecule assay. Molecules influence the EB from Day 2-4 and again from Day 6-10. These developmental time frames influence both the early formation of cardiac mesoderm and late stage cardiomyocyte differentiation, offering a total coverage of the influence of a novel small molecule on cardiomyocyte formation.

4.7 Initiating Directed Differentiation for Cardiomyocytes Day 0

In order to control the differentiation of embryonic stem cells to differentiated mesodermal multipotent cardiac progenitor cells first they are treated with a series of specific cytokines. Before this can be achieved their differentiation is controlled by forming EBs, similar to the HD model but within a suspension, which allows them to form the three germ layers. The suspension model offers less even and uniform EBs than the 96-well plate but with a larger total number of cells and thus offers scalability. The cells are distributed at a density of 750,000 cells per 10 ml of SFD (with MTG and Ascorbic acid). Cells are distributed throughout the plate and incubated for 48 hours at 37 ℃, 5% CO2 and 90 % humidity (Figure 6).

4.8 Directed Differentiation of Cardiomyocytes Day 1

24 hours after plating, the cells were viewed under the microscope. EB formation and health of the EBs was recorded. An EB is a mass of cells which grow in a spatial arrangement and differentiate based on the local position of cells within the mass. A healthy EB should be; evenly round, clear, not dark or opaque, whole without splintering of cells away from it, and after 24 hours the EBs should be of uniform size. Larger EBs are evidence of poor

trypsinization during Day 0, smaller EBs or no EBs are evidence of over trypsinization, cold media, or harsh physical treatment of cells.

4.9 Directed Differentiation of Cardiomyocytes Day 2: Induction of Mesoderm

Day 2 is the induction time point were the cells are directed towards a mesodermal fate by a 40-43 hour temporal exposure to cytokines BMP4, Activin A, and VEGF. The cells were plated in 10 cm sterilin dishes. Collecting the EBs was done by gently swirling the dish 5-10 times and then EBs were pipetted up while simultaneously tilting the dish. The pipette was turned to the lowest setting, to prevent fracturing the EBs during collection. 5 ml of IMDM was added to the dishes and used to gently wash the dish at a high angle twice with the total 15 ml of media and cells transferred to a 15 ml tube. The additional wash must be gentle as your target is to collect EBs left behind from the initial collection but no single cells. The single cells do not contribute to the EBs and will reduce the efficiency of the differentiation because they do not go through the same controlled EB differentiation. Collected cells are then centrifuged at a slower rate, again this slower centrifuging is utilized to remove any single cells. At slower speeds only the larger collated cells will reach the bottom of the tube while the single cells will remain in suspension and be removed during aspiration.

With the SFD and IMDM aspirated 1 ml of trypsin was added. With the trypsin added, the tube was flicked until the EBs swirl away from the bottom. Cells are immediately place in a 37℃ water bath for 1-2 minutes. After one minute, or 30 seconds, the tube was flicked again to mix the EBs. After 1 or 2 minutes remove the tube and EBs are pipetted up and down three times. With MEF media added the cells are pipetted to mix the MEF and trypsin. 8 ml of MEF media was added to ensure total inactivation of trypsin. The total 10 ml was then mixed. The inactivated trypsin and cells was then centrifuged and the media aspirated. The cells are then washed twice with IMDM, centrifuging and aspirating the media each time.

With the cells now washed free of all residual serum, from the MEF media, they are resuspended into SFD media containing MTG, Ascorbic acid, BMP4, VEGF, and Activin A. The cytokines were titrated to find the correct concentration depending on the cell line or passage being used. All cytokines must be stored in the -80℃ freezer and only thawed immediately before distributing them to the media. The media should be room temp but not 37℃ as the half-life of BMP4 and Activin A are greatly reduced at 37℃. Keeping the media at

room temp and allocating the cytokines immediately after thawing will produce the optimal experimental conditions. With the cytokines added to the media, add 750,000 cells per 10 ml of media for the desired number of plates. Each plate yields approximately 1.5 million cells for Day 4.

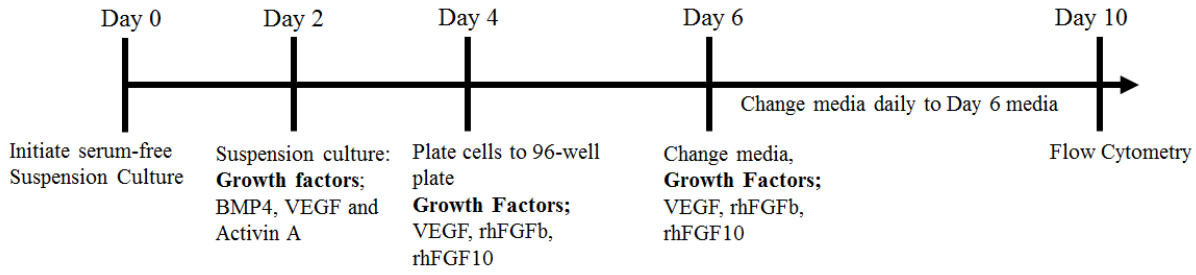

Figure 6: Timeline for Directed Differentiation and Growth Factor Layout. This depiction, without small molecule screening, shows the stepwise directed differentiation of pluripotent stem cells to cardiomyocytes via a cytokine induced differentiation.

4.10 Directed Differentiation Day 3, an Optimal Induction

Day three EBs should look similar to Day 1 EBs; small, transparent, little blebbing or fracturing, and spherical. The EBs should also be gently spread out in the dish as clumping of EBs can cause fusion of EBs and lead to a reduced cardiomyocyte yield. This can easily be achieved by placing the plates gently on a flat, sterile surface and moving them in the standard nautical compass, first North to center, then East to center etc... not swirling the plates or move them in a centripetal motion as it can cause the EBs to clump together and potentially fuse, cyclone effect.

4.11 Directed Differentiation Day 4, Two Ways Forward

Day 4 in the embryonic developmental pathway of *in vitro* EBs during directed differentiation for cardiomyocytes means that differentiated cells have passed through the three germ layers and are expressing cardiac progenitor markers. These cells are expressing a number of cell surface receptor markers as well as intracellular protein markers. For experimental purposes two things can be done, cells can be plated directly to 96 well F-bottom plate at

125,000 cells per well or sorted for cardiac progenitor cell surface markers FLK1 and/or PDGFRα using the MACS system.

4.12 Directed Differentiation of Cardiac Progenitor Cells Day 4, Plating to Monolayer

The EBs were collected from sterilin dishes in the exact same way as described in Day 2. Instead of resuspending the cells in SFD before counting, the cells are resuspended in Maintaining media, containing VEGF, hFGFb, hFGF10, and ascorbic acid. The cells are counted to determine the number of desired wells needed. Add the number of cells, 125,000 cells per well, to a new tube containing 250 µl of Maintaining media per desired well. Cells are pipetted at 250 µl of the mixture to each well of a gelatinized F-bottom 96-well plate. The cells are settled for 15 minutes undisturbed before moving them directly to the incubator.

4.13 Directed Differentiation of Cardiac Progenitor Cells Day 4, FLK1 MACS

Following the same steps as "Plating to Monolayer" the cells are counted and instead of plating all cells only 4-6 wells for an unsorted control were plated for later Flow Cytometry. Cells were resuspended in 5 ml of IMDM. A 40 µM cell strainer was used to strain the cells into a 50 ml tube. The filter was pre-wet with 2 ml of IMDM to reduce the stress on the cells during straining. The cells are resuspended into 5 ml of IMDM and added to the cell strainer. The cell strainer was rinsed with 8 ml of IMDM to rinse all the single cells through the strainer. The cells were then transferred to a 15 ml conical tube and centrifuge. The IMDM was aspirated and the cells were washed one time with 10 ml of room temperature PBS. The cells were then centrifuged and the PBS aspirated. With the cells pelleted, 80 µL of FLK1 Buffer was added and 20 µL of CD309 conjugated to biotin (FLK1 Microbeads) was added. Cells were mixed 8-10 times to ensure a single cell suspension. Any cell aggregates will cause non-specific cell sorting and reduce the efficiency of the experiment. Cells are incubated with microbeads for 10 minutes, at 4℃, in the dark. Then the cells were washed with 2 ml of FLK1 Buffer: centrifuged, buffer was aspirated and cells are resuspended in 80 µL of FLK1 Buffer. 20 µL of Anti-biotin Microbeads was added next, mixed well and incubated in the 4℃ refrigerator for 15 minutes, in the dark. The cells are washed again and magnetically separated (Figure 7).

To separate labeled cells the OctoMACS (Miltenyi Biotec) separator and magnet were used along with the MS magnet column. The magnet column was loaded with FLK1 Buffer. The FLK1 Buffer must be pre-cooled to 4℃ before the experiment and was de-gassed to ensure no small air bubbles clog the magnetic filter. The buffer was degassed using a sonicator containing Milli-Q water for 30 minutes. It was also important to not allow the buffer to exceed 25℃ as this will damage the buffer by inactivating the bovine serum albumin (BSA) it contains. The magnetic column was rinsed with 500 µL of buffer, the cells were resuspended in 500 µL of buffer and added to the column. Lastly the cells are washed three times with 500 µL of buffer.

The cells which pass through the magnetic column are negative for the selection marker. The magnetic column can be removed and using a new tube, 1 ml of buffer was added to the column to elute the positive cells. With the two fractions of cells separated the cells were resuspended in an equal volume of buffer. For a FLK1 only MACS, the supernatant was removed and the cells were resuspended in Maintaining Media and counted. The cells were plated to a 96 well F-bottom gelatinized plate with 125,000 cells per well. The cells are allowed to settle undisturbed for 15 minutes before being moved to the 37℃ incubator.

4.14 Directed Differentiation of Cardiac Progenitor Cells Day 4, PDGFRα MACS

The cells were pelleted and resuspended in 80 µL of PDGFRα buffer. 20 µL of Multisort Release solution was added and incubated in 4℃ in the dark for ten minutes. This action removes the FLK1 microbeads from the cells. The cells were washed again in PDGFRα buffer and centrifuged. Next the cells were resuspended in 60 µL of buffer, 20 µL of Multisort Stop solution, and 10 µL of PDGFRα conjugated microbeads. Incubated in 4℃ in the dark for 15 minutes. The cells were finally washed in buffer, centrifuged and resuspended in 500 µL of PDGFRα buffer. Two new magnetic columns were used, one for the FLK1+ positive fraction and one for the FLK1- fraction (Figure 7) and were separated following the same guidelines as previously defined. Sorted cells were washed in PDGFRα buffer and centrifuged. The cells were resuspended in Maintaining Media, counted, and plated at 125,000 cells per well to a 96 well F-bottom plate. The cells were allowed to settle for 15 minutes and then moved to the 37℃ incubator. This procedure takes about 4-5 hours of intensive, non-stop cell culture work.

Proper preparation for this experiment will improve the experimental results as the long time threatens the health and viability of the cells.

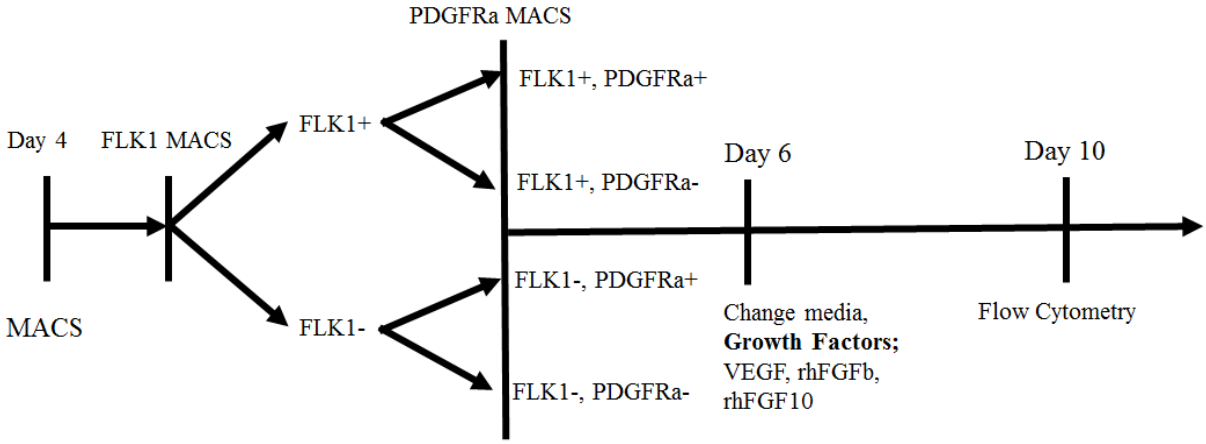

Figure 7: MACS experimental timeline shows the magnetic separation of selected cell populations based on magnetically selective microbeads. The populations are all separated on Day 4 in a two part, serial, MACS utilizing FLK1 first and PDGFRα second.

4.15 Culturing Cells Day 6 to Day 10

With the cells sorted, or the cells plated without sorting, cells were incubated for two days without disturbing. After two days the media was changed to new Maintaining Media, Day 6. On Day 7, for a molecule screening experiment, molecules were added to the media by diluting the molecule 1:1000 in media (Figure 8). Subsequently changing of the media every morning by aspirating the media with a glass pasteur pipette covered with a plastic pipette tip and replacing media was done. Observable beating occurs late Day 7 or Day 8. On Day 10 the culturing of the experiment was completed. For a successful differentiation or molecule assay fluorescent pictures were taken to catalog the intensity of the eGFP expression. As the eGFP expression is coupled to the Myl2 gene expression, the activation of this target gene can be observed via the intensity of the green fluorescence detected. Included in the molecule screen was a negative control which was retinoic acid 2.5 µM or GSK3 inhibitor 3 µM which slows the differentiation of cardiomyocytes and provided a gating reference for downstream applications. If culturing the cells beyond Day 10 the media was switched to RPMI media with BSA which will slow down the proliferation of non-cardiomyocyte cell types. The maximum culturing for

in vitro cardiomyocytes in 96 well F-bottom plates was 18 days as the beating cardiomyocytes detached from the plate and lose viability.

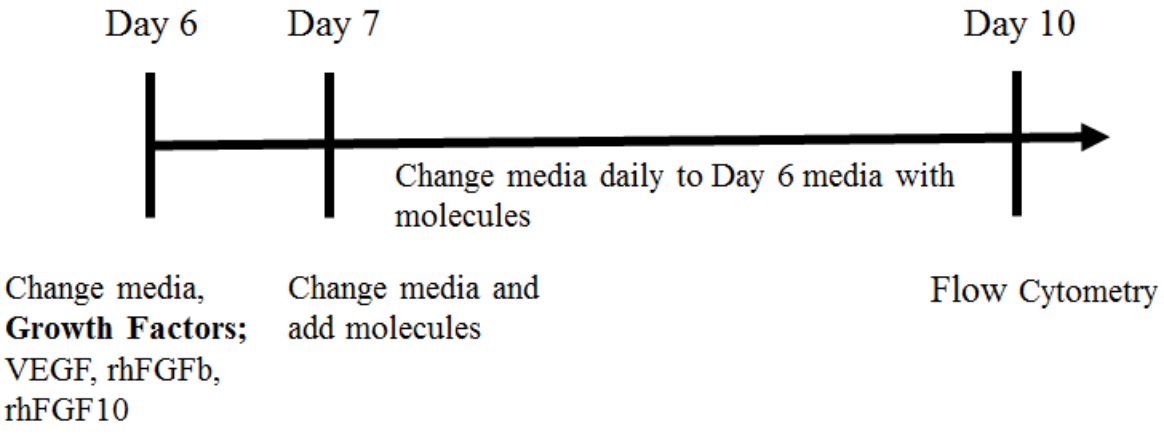

Figure 8: Cardiogenic molecule assay using MACS cells. Once cardiac mesoderm has been induced from Day 0-4 the cell populations can be screened against novel small molecules Day 7-10. This assay effectively allows the screening of molecules against late stage cardiomyocyte differentiation and offers a drug screen against a cardiomyocyte enriched population.

4.16 Quantifying Cells using Flow Cytometry

Cells were quantified using the BD Accuri C6 Sampler which allows either analysis of live cells through eGFP detection or fixed cells through cTnT staining (647 fluorophore). The media was aspirated using a glass pasteur pipette with a plastic pipette tip. 100 µL of trypsin was added and incubated for 5 minutes in the 37℃ incubator. Cells were removed by gently stirring the cells and scraping the monolayer apart with a plastic pipette tip. Cells were then placed back in the incubator for 4 minutes at 37℃. After incubation the cells were pipetted up and down with only trypsin in the well to finalize the dissociation. 150 µL of Fetal Bovine Serum (FBS) was added to quench the trypsin and the cells were pipetted up and down 5 times to quench the cells. The contents of the well was then transferred to a 1.5 ml microcentrifuge tube, and centrifuged at 300xg for 3 minutes to pellet the cells. For live cell flow cytometry the cells were washed once with 1 ml of PBS and resuspended in 300 µL of PBS. The cells were stored on ice until flow cytometry of live cells for Myl2-eGFP detection.

For fixation of cells and flow cytometry using cTnT, the media was aspirated and the cells were resuspended in 500 µL of 4% Paraformaldehyde (PFA). The cells were then

incubated in the dark for 25 minutes at room temperature and centrifuge for 3 minutes at 300xg. The PFA was aspirated and the cells were washed with 1 ml of PBS. After washing the cells were resuspended in 300 µl of PBS.

4.17 Flow Cytometry for Intracellular Cardiac Troponin T

Fixed cells can be stained with cTnT up to three weeks after fixation. Before staining two buffers were prepared to perform the staining. FACS buffer with 4% FBS in sterile PBS and FACS buffer with 4% FBS, 0.5% Saponin in sterile PBS. Saponin is a mild detergent that acts as a permeabilizing agent. With the cells in PBS the cells were pelleted and the PBS was aspirated. The cells were washed twice with FACS buffer with Saponin. FACS buffer was aspirated from the cell pellet and the cells were resuspended in 200 µL FACS buffer with Saponin containing 1:100 cTnT antibody. The antibody for cTnT was incubated for one hour at room temp. The cells were then pelleted and media aspirated. 2 FACS buffer with Saponin washes were performed. The media was aspirated from the cell pellet and the pellet was resuspended in 200 µL of FACS buffer with Saponin containing a 1:200 dilution of secondary antibody (594 or 647). The cells were then incubated in the dark at room temperature for 30 minutes. After this step the cells were kept in the dark for all techniques and at all times to optimize the flow cytometry read out. The cells were finally washed twice with FACS buffer with Saponin and once with FACS buffer without saponin. The cells were then resuspended in 300 µl of PBS and store at 4℃ or on ice until flow cytometry.

4.18 Quantifying Cells for Fluorescent Microscopy

Cells from all experimental models can also be imaged via fluorescent microscopy using fluorophore conjugated antibodies. Here cells were stained for cardiac troponin T (cTnT), a marker of sarcomere protein, and myosin fibril 20 (MF-20) a marker for myosin. On Day 10 of cardiomyocyte differentiation instead of fixing cells in 4% PFA they were split to glass coverslips in a 12 well plate. The cells were taken from a 96 well F-bottom plate and transferred via trypsinization: add 50 µL trypsin and incubate for 10 minutes at 37 ℃. Next cells were removed from the incubator and pipetted up and down 6 times, gently, in only trypsin. 200 µL of FBS was added to the wells and pipetted 5 times. The quenching of trypsin was done with a higher ratio of FBS, 1:5 in this case, because the trypsin will also be transferred

to the new well instead of removing it. The contents of the 96 well F-bottom plate was transferred to one well of the 12 well plate containing RPMI.

4.19 Coverslip staining

Staining cell cultures for quantification of cell type was achieved through a basic immunocytochemistry technique utilizing BSA blocking, Triton 100x for permeabilization, and primary antibody staining overnight. For cTnT and MF20 a concentration of 1:100, antibody:BSA solution, was used. The samples were incubated at 4℃ on a rocker for 16 hours or overnight. Secondary staining was 1:200 for one hour and samples were stored for up to two months in 4℃ in the dark.

5. Results

In order to establish a consistent molecule assay first the formation of cardiomyocytes in the spontaneous molecule assay was confirmed. Utilizing the hanging drop model functional cardiomyocytes can be observed, beating, *in vitro* with cTnT staining (Figure 9). As cTnT is required for sarcomere formation and the sarcomere provides the structural basis for contractility in cardiomyocytes, its presence is a marker for functional cardiomyocytes. In addition beating foci can be viewed under the brightfield microscope and videos can be made to show the beating. From the experiments all Myl2-eGFP+ cells are discovered to be also cTnT+. This EB model spontaneously yield approximately 1% beating cardiomyocytes consistently and across several independent experiments. To visualize beating cardiomyocytes EBs were stained on coverslips. Figure 9 shows results from staining on coverslips which confirms the presence of cTnT positive and Myl2 positive cells. In addition to staining on coverslips Figure 10 shows flow cytometry results for detection of Myl2-eGFP+ cells for a selection of molecules with cardiogenic effects which increase cardiomyocyte purity in the spontaneous differentiation model to greater than 2.0%. Figure 10 also shows selected cardiosuppressive molecules which decrease cardiomyocyte yield below 0.5%. Using DMSO as the baseline for detection 1% was identified as the cardiomyocyte purity in DMSO control. Molecules which showed cardiogenic effects in the EB model were moved along the pipeline to the directed differentiation model.

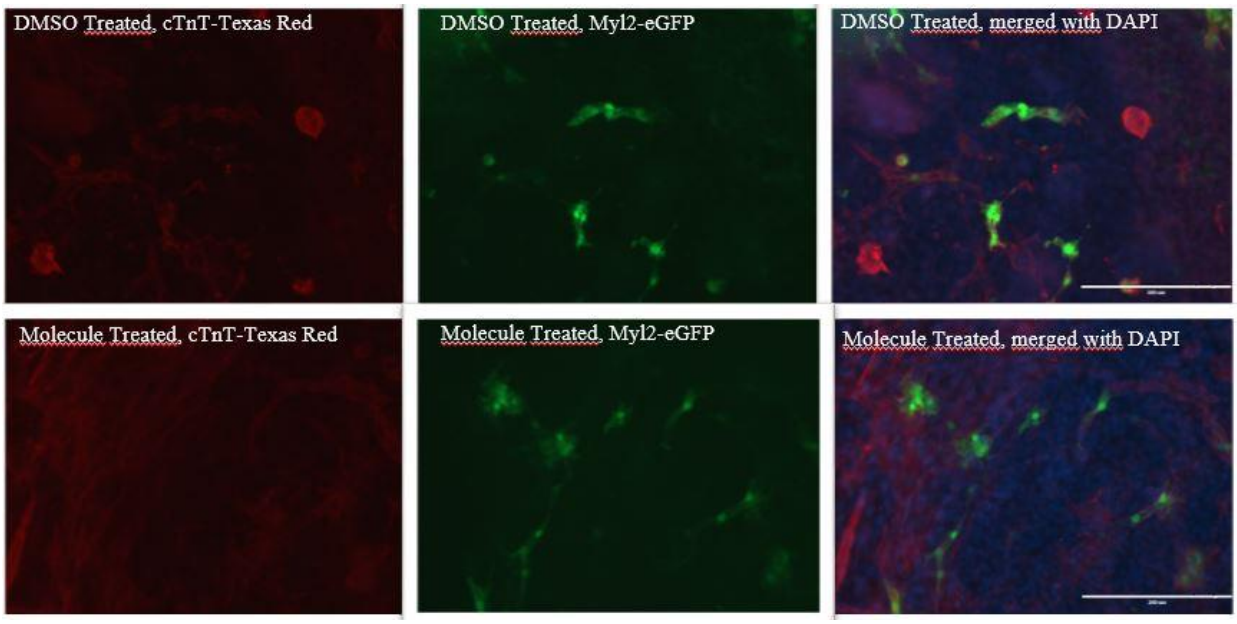

Figure 9: Staining from spontaneous differentiation molecule assay using the hanging drop. A spontaneous differentiation assay with molecules influencing the EB Day 2-4 and Day 6-10. The pictures are made using fluorescent microscopy with green representing eGFP from Myl2 protein, red representing Texas Red conjugated to Cardiac Troponin T, and blue representing nucleus stained with VectaShield for DAPI. Gain = 7.0X, exposure time = 780 MS, mag 20X

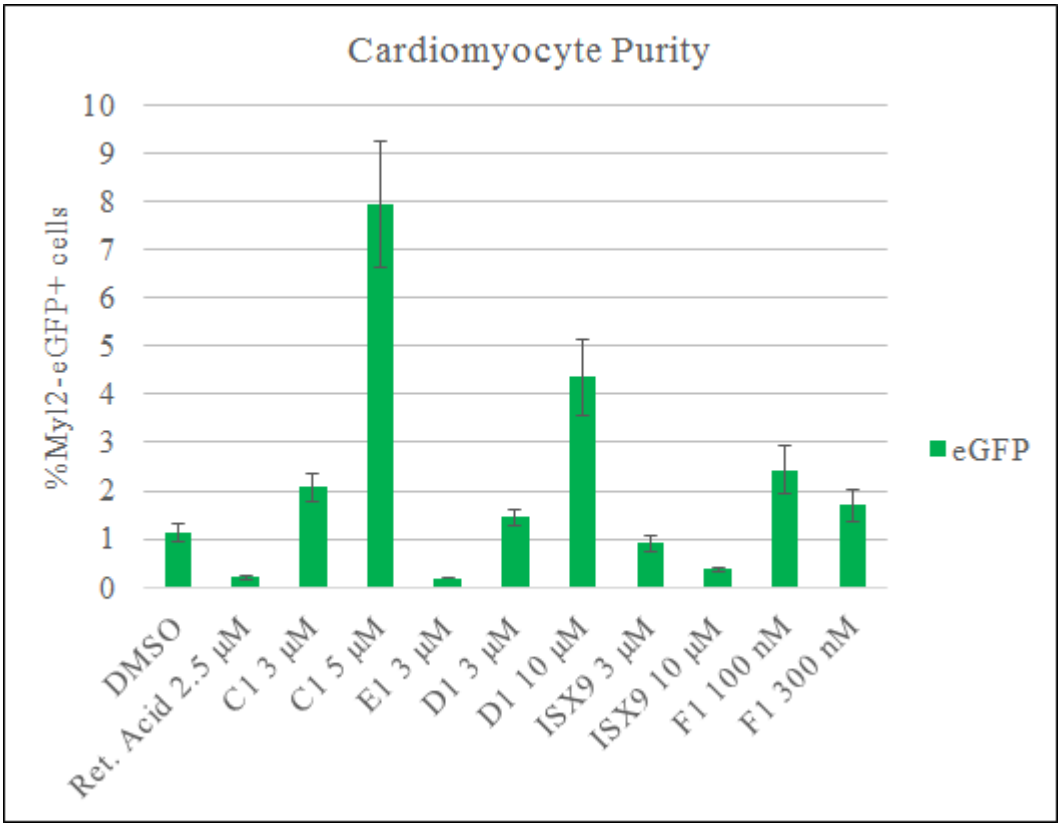

Figure 10: Cardiomyocyte purity from an EB molecule assay. A selection of molecules from flow cytometry results from a Spontaneous Differentiation Molecule Assay with molecules present from Day 2-4 and Day 6-10. Flow cytometry on Day 10. Flow Cytometry on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=3, error bars (SEM)

Once molecules have passed the spontaneous differentiation assay by showing no toxicity at the desired concentration and showing cardiogenic effects, greater than 2% Myl2-eGFP + cells, they are tested in a cardiomyocyte enriched environment via the directed differentiation model. This model utilizes a directed differentiation technique and yields ≅20% Myl2-eGFP+ cardiomyocytes. Figure 11 shows DMSO control around 15% cardiomyocyte purity. In this model an increase in the cardiomyocyte purity is observed through an increase in Myl2-eGFP+ cells in the Wnt Inhibitor at 1.2 µM concentration. This inhibitor effectively down regulates the Wnt pathway on Day 7 and increases the *in vitro* cardiomyocyte purity by around 4%-5%. The use of GSK3 inhibitor at 3 µM in this molecule assay is an effective negative control. In addition the C1 compound shows an increase in ≅5% cardiomyocyte purity.

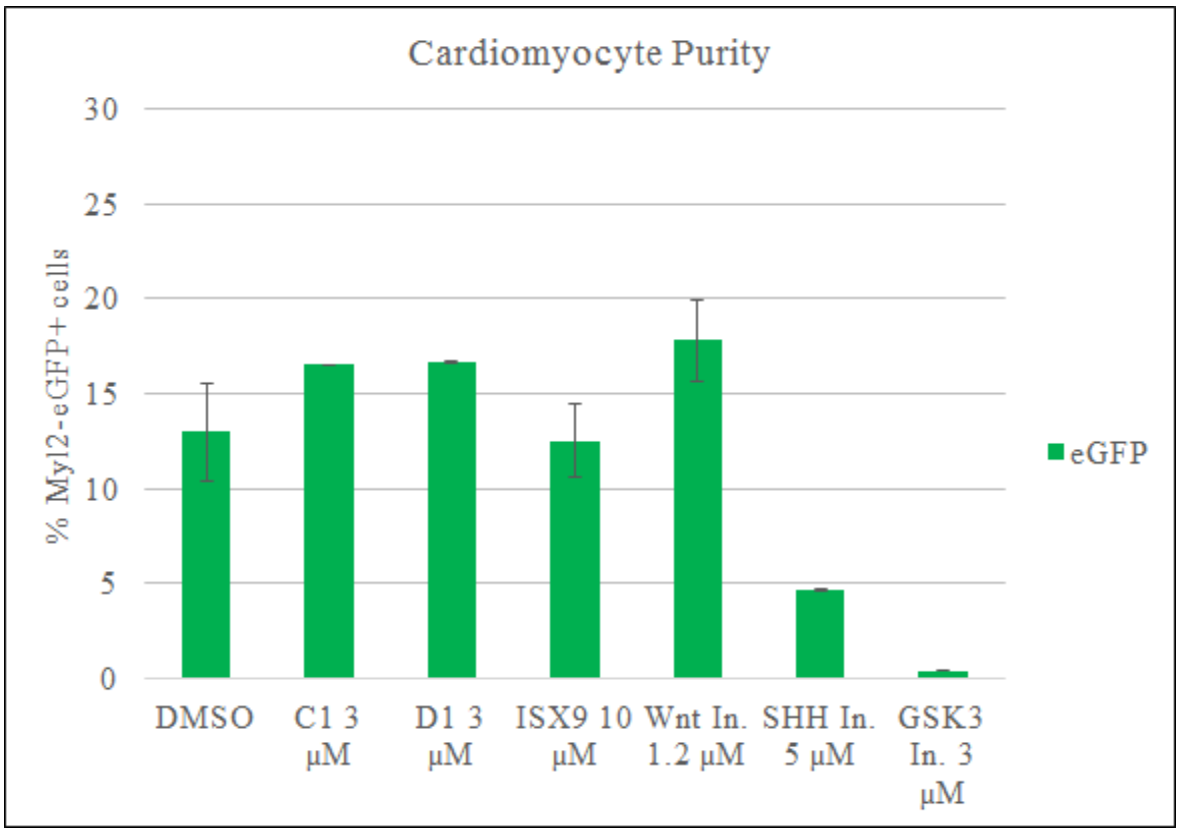

Figure 11: Directed differentiation assay showing molecules screened against a cardiomyocyte enriched population. Molecule addition Day 7-9. Flow cytometry on Day 10. Flow Cytometry

on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=3, error bars (SEM)

To increase the relevance of the drug screen model, cells are sorted with FLK1 and PDGFRα to optimize the assay for a mesoderm molecule assay. Figure 12 shows fluorescent images of Myl2-eGFP positive cells in a monolayer 96 well F-bottom plate. Sorted cells can be treated with molecules on Day 7-10 and then measured via flow cytometry for either an increase or decrease in cardiomyocyte purity. To establish this model the cells are first sorted with FLK1 and PDGFRα separately. It was determined that the FLK1 sort yields better results than the PDGFRα sort based on fluorescent imaging. Fluorescent imaging of MACS cells shows a clear separation of Myl2-eGFP populations in the FLK1 sorted cells, while the PDGFRα shows enrichment of Myl2-eGFP+ cells but an incomplete separation.

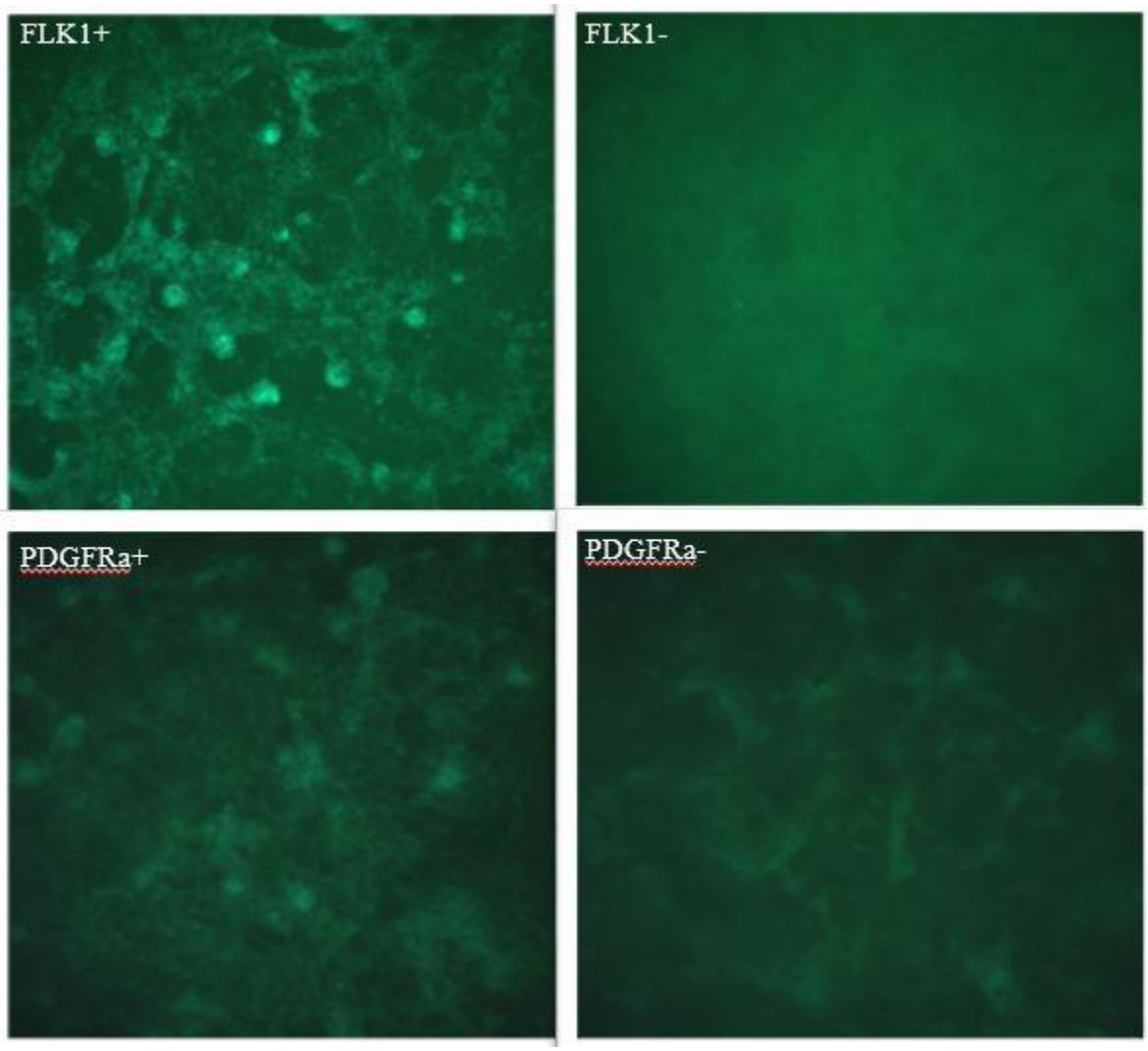

Figure 12: Proof of concept for MACS FLK1 (CD309) and PDGFRα (CD144α). Proof of concept from the initial pilot experiment shows two separate cell sorts using microbead conjugated antibodies. All images show Myl2-eGFP+ cells with; gain = 7.0X, exposure time = 460 MS, magnification = 10X

Due to the incomplete separation of Myl2-eGFP fractions in the PDGFRα MACS it was decided to continue with only FLK1 MACS cells. Interestingly the FLK1+ population yields an increase in Myl2-eGFP+ cells and a strong platform for a mesoderm molecule assay. Figure 13 shows flow cytometry results for Myl2-eGFP+ cells on Day 10 after cell sort. Due to some variation in the antibody binding the FLK1- contains a small percentage of cardiomyocytes, while FLK1+ samples show an enriched population of cardiomyocytes. The sorted cells are derived from a directed differentiation which has ≅20% Myl2-eGFP+ cardiomyocytes (unsorted) while the enriched cardiomyocyte cultures (FLK1+) yield 25% Myl2-eGFP+ cardiomyocytes.

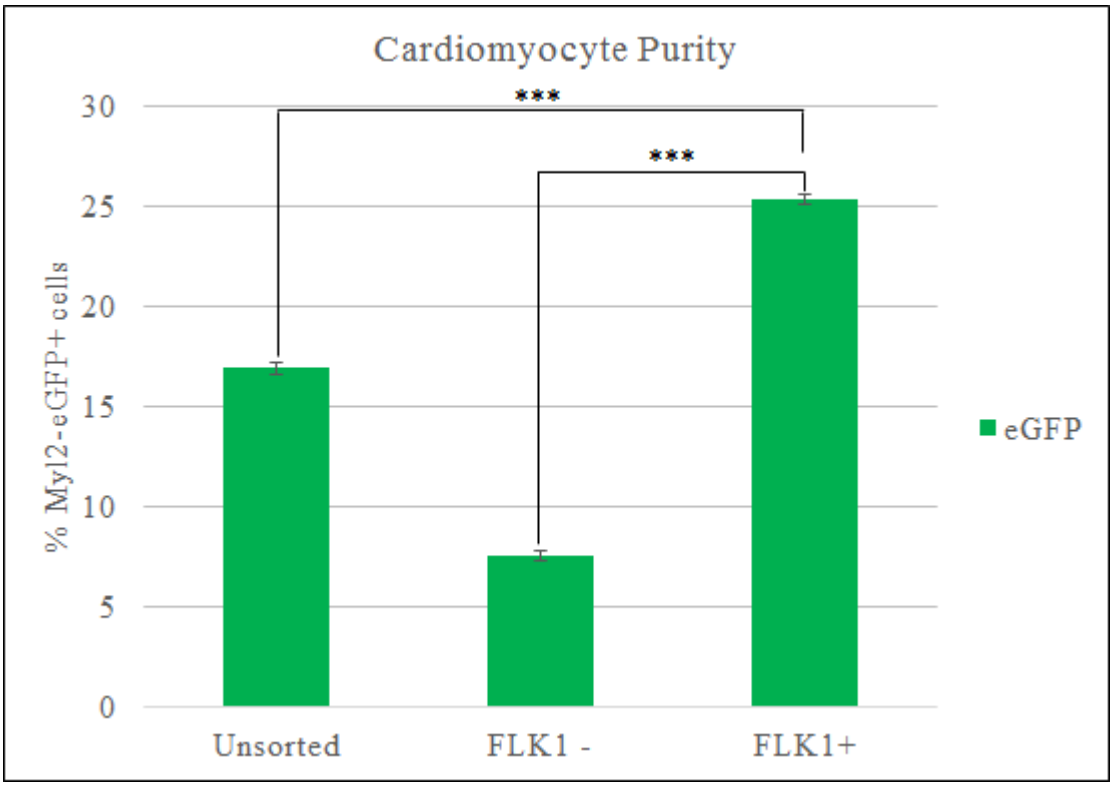

Figure 13: Cell sort for FLK1 multipotent cardiac progenitor cells. Flow cytometry analysis from; unsorted, FLK1- and FLK1+ fractions reveals significant enrichment of cardiomyocytes in the FLK1+ cell population $p<0.005$ (≅25%), when compared to the unsorted (≅15%) and FLK1- (≅5%) . However some detectable Myl2-eGFP+ cells in the FLK1- population (≅5%) indicate nonspecific cell sorting. Flow cytometry on Day 10. Flow Cytometry on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=3, error bars = SEM

Continuing with the exploration of the MACS labeling system the PDGFRα cell sort was also quantified using flow cytometry. Figure 14 shows PDGFRα+ cells on Day 4, when the FLK1 sort is also performed, and on Day 10. In line with the current publications it can be observed that Day 4 expression of PDGFRα is around 60 % PDGFRα-APC+ conjugated cells while Day 10 expression is almost non-existent. This result supports our use of FLK1 and PDGFRα as appropriate cell surface markers to use for the Day 4 MACS.

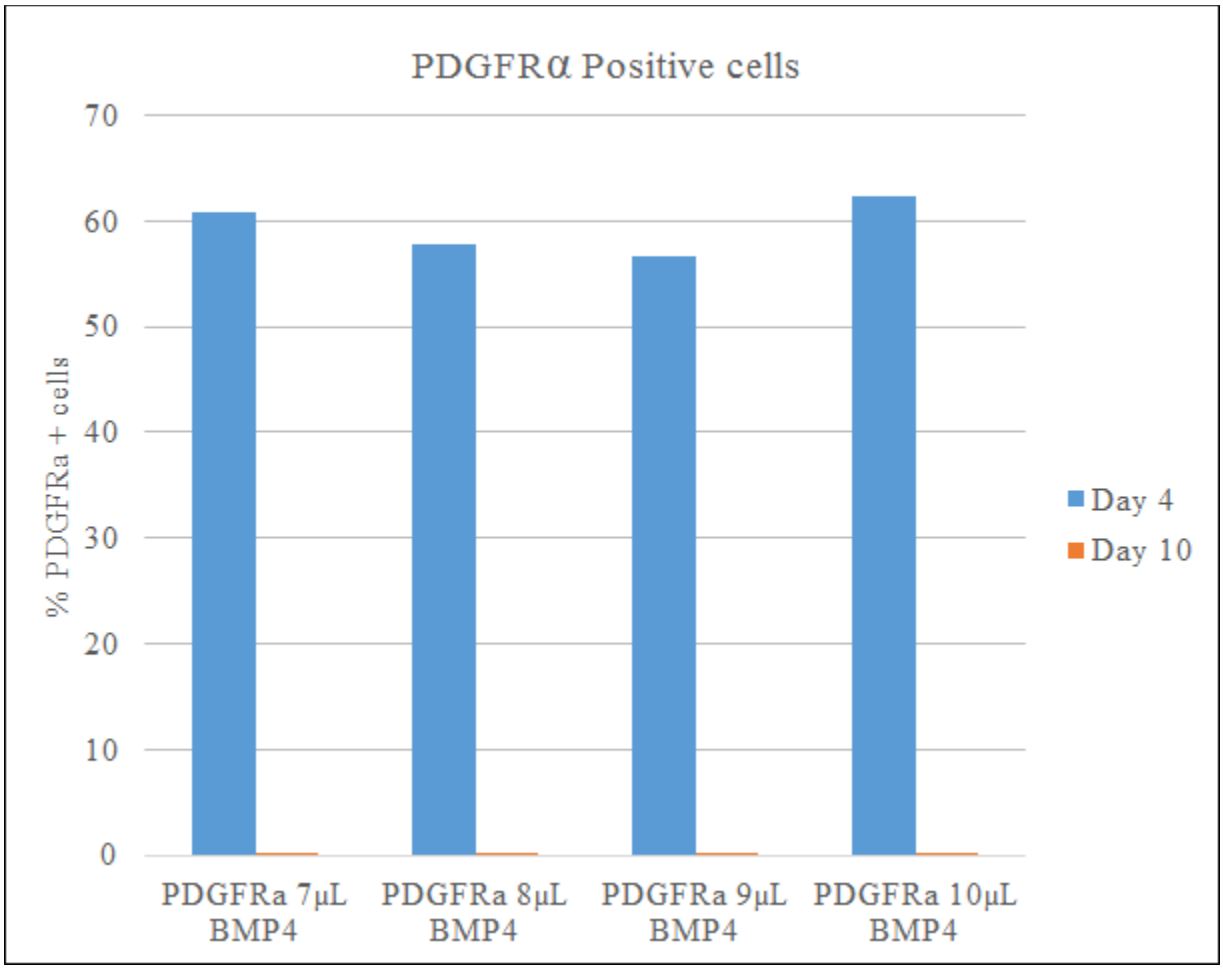

Figure 14: PDGFRα flow cytometry results from Day 4 and Day 10. PDGFRα+ cells are labeled for flow cytometry which shows a peak of expression on Day 4 when cells are sorted. BMP4 titrated for optimizing experiment. Flow cytometry at the Biomedicum FACS core unit, Helsinki. 10,000 cells counted as a minimum threshold. N = 1

To investigate the possibility of using a serial MACS system for molecule screening a FLK1 sort was followed by a PDGFRα sort to isolate cardiac progenitor cells. Figure 15 shows

the enrichment of Myl2-eGFP+ ventricular cardiomyocytes in the FLK1+, PDGFRα + cell fractions when compared to the unsorted fraction or the FLK1- PDGFRα- fraction.

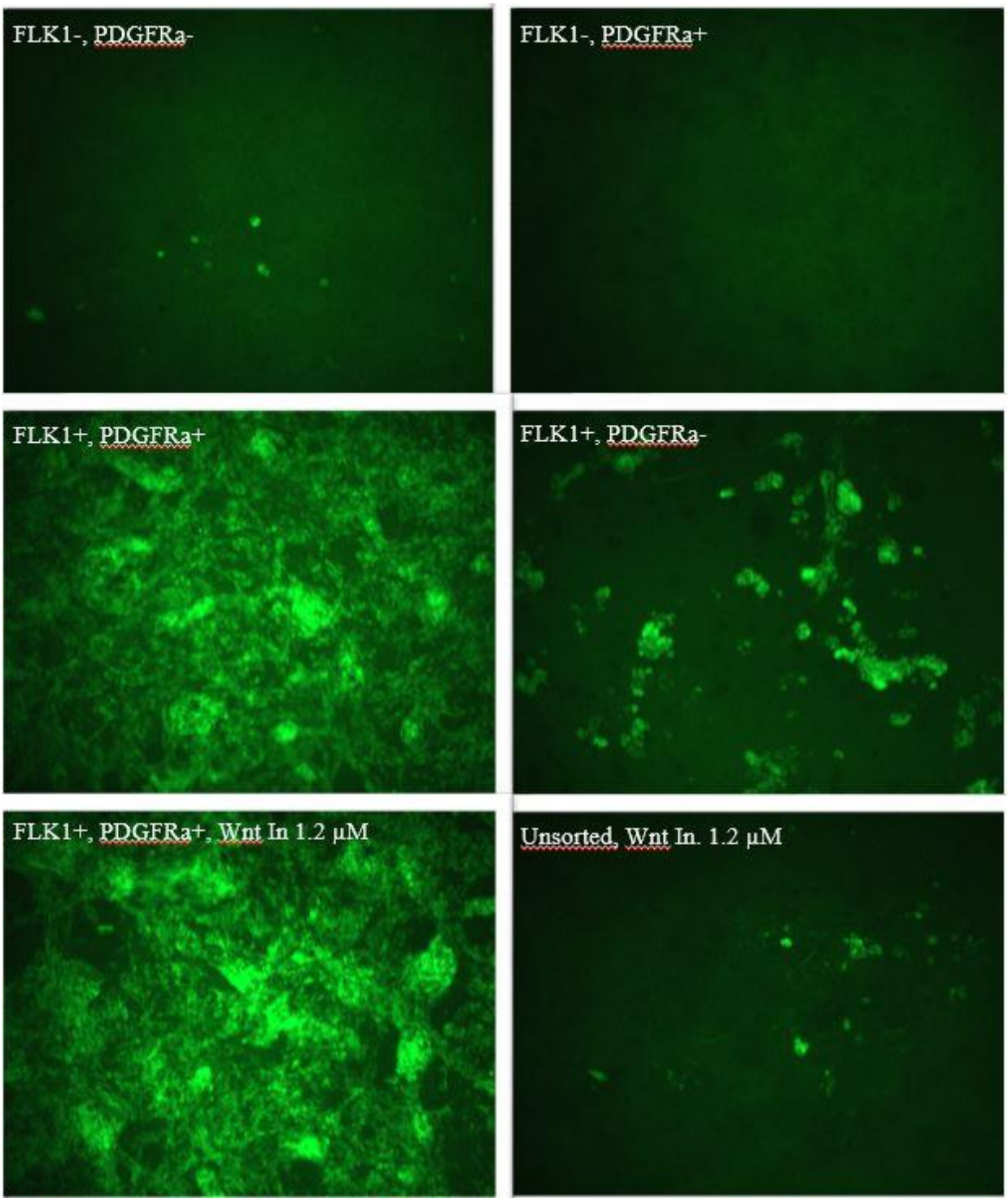

Figure 15: MACS serial sort for selective markers. A double sort using first FLK1 and then PDGFRα shows an enriched population of Myl2-eGFP+ ventricular cardiomyocyte in the

FLK1+, PDGFRα+ samples. All samples contain 125,000 cells at plating and no conditions were toxic. Exposure time 160 MS, gain 7X, mag = 10X

Flow cytometry analysis of the FLK1 and PDGFRα sorted cells is consistent with the fluorescent imaging analysis and shows an increase in the overall cardiomyocyte yield in the FLK1+, PDGFRα+ fractions (Figure 16). Although yielding a high cardiomyocyte purity the double MACS system does not yield enough total cell numbers to have enough replicates in a cardiogenic molecule assay based on the target model design. From a starting culture of ≅20 million cells only ≅300,000 multipotent FLK1+, PDGFRα+ cardiac progenitor cells are recovered. Due to the success of FLK1 only MACS and the low yield from the serial FLK1 and PDGFRα, experiments continued that use only the FLK1+ cell sort made a better assay platform for testing small molecules. The high cardiomyocyte purity in these assays provides an attractive platform for testing small molecules in a population derived largely from CPC, unfortunately low cell yield prevents the number of replicates needed for drug screening.

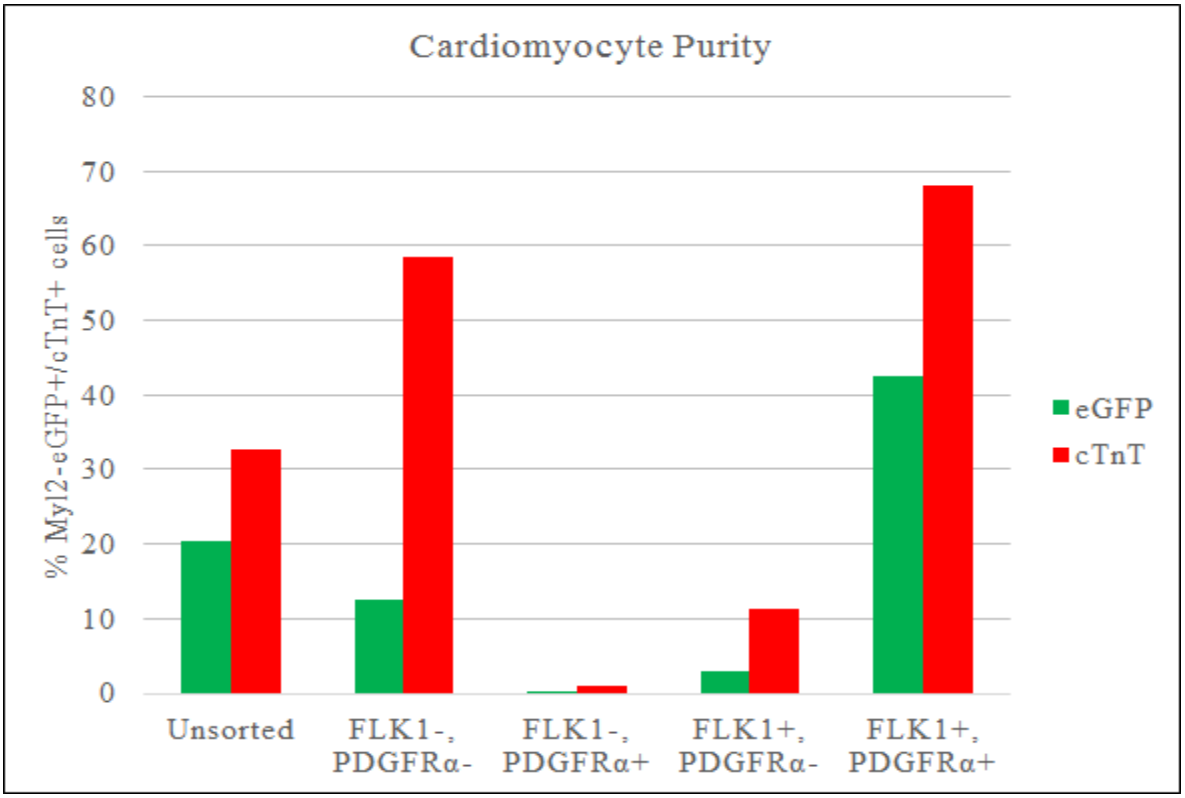

Figure 16: Flow cytometry results from FLK1 and PDGFRα serial sort. Flow cytometry analysis of MACS cells shows an enrichment of Myl2-eGFP + cells in the double positive samples, FLK1 + and PDGFRα +. Depletion of Myl2-eGFP + cells is also observable in all other selective conditions when compared to unsorted controls. Flow cytometry on Day 10.

Flow cytometry on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=1

After confirming an enrichment of cardiomyocytes in the FLK1+ fraction over three experiments drugs and novel molecules are screened in the MACS sorted cells. Using the Myl2-eGFP reporter line for ventricular cardiomyocytes yielded more cardiomyocytes in the FLK1- population compared to the FLK1+ population. The FLK1- population shows a further increase in cardiomyocytes when treated with Wnt Inhibitor 1.2 µM and SHH inhibitor 3 µM. In the FLK1+ population there was an increase in cardiomyocyte purity with the Wnt Inhibitor 1.2 µM, C1 20 µM and the ISX 9 10 µM.

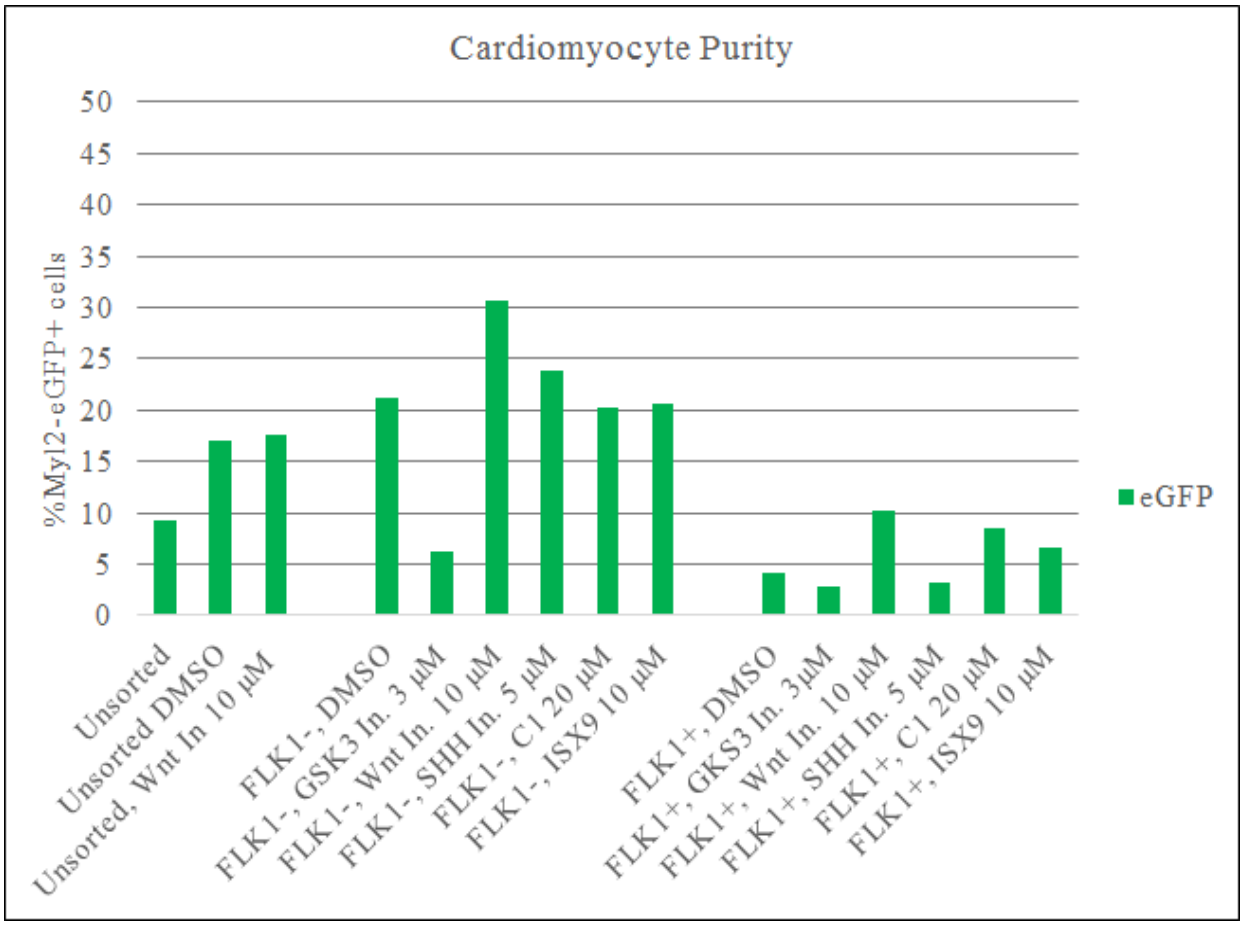

Figure 18: Cardiogenic molecule assay using MACS system to sort for FLK1 +/- cell fractions. Molecule addition on Day 7-10. Assay shows a higher concentration of Myl2-eGFP+ cells in the FLK1- population. Molecule effects on cardiomyocyte purity can be compared to the unsorted and the FLK1-/FLK1+ DMSO controls. Flow cytometry on Day 10. Flow Cytometry on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=1

Further testing of the molecules using the cTnT staining yielded similar results with cTnT positive cardiomyocytes detectable in the FLK1- and FLK1+ fractions. In this assay the C1 molecule was toxic in FLK1- at both 5 µM and 10 µM. It was observed that there was no increase in cardiomyocyte purity in the FLK1- population. In the FLK1+ fraction it was shown that C1 5 µM and C1 10 µM, and Wnt Inhibitor 1.2 µM increased the cTnT+ cardiomyocyte purity.

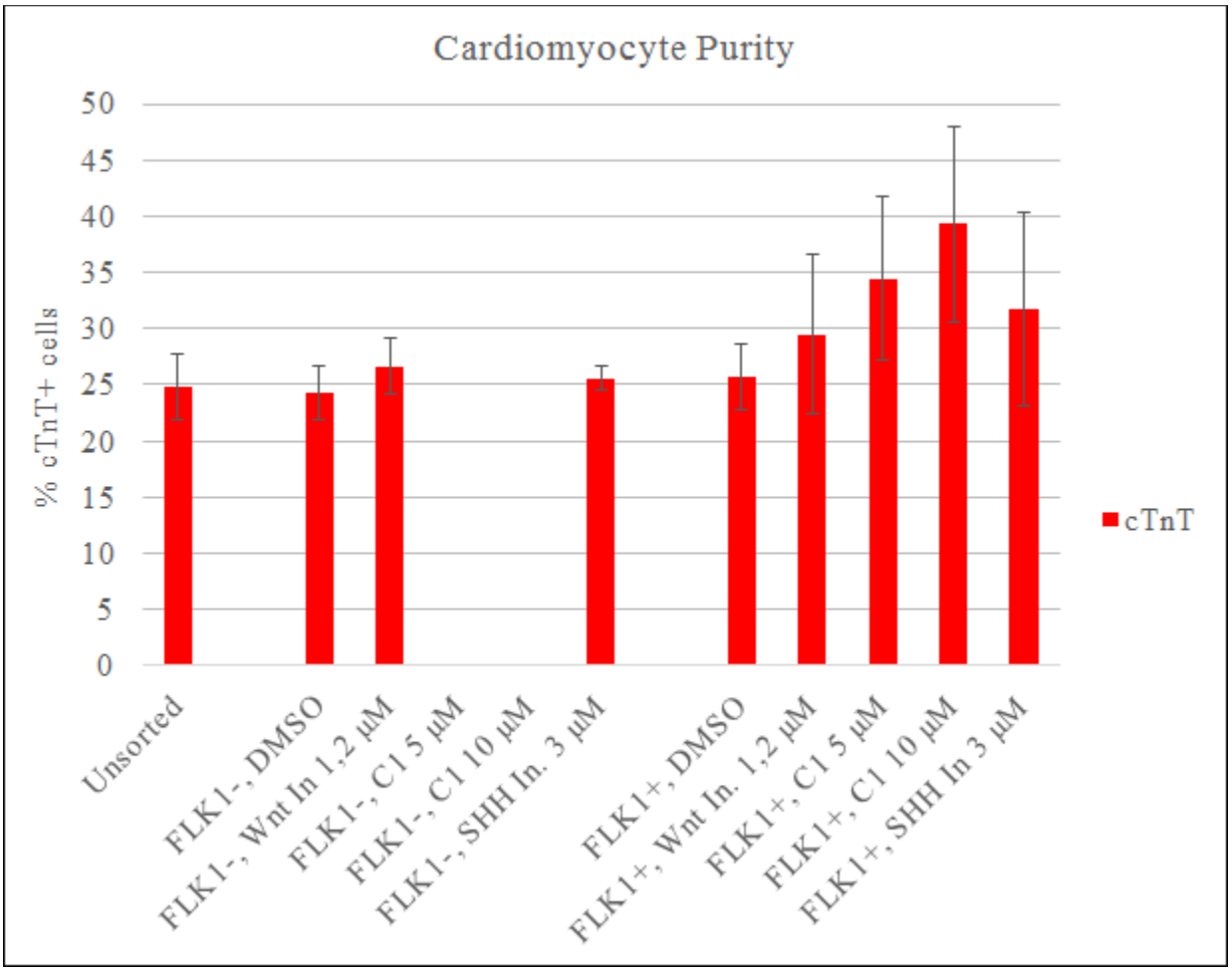

Figure 19: Mesodermal molecule assay using cTnT detection. MACS sorting for FLK1 +/- cell fractions with molecules added Day 7-10. Flow cytometry on Day 10. Flow cytometry on the BD Accuri C6 sampler with 10,000 cells counted as the minimum threshold. N=3, error bars (SEM)

Molecules screened in the MACS molecule assay represent the highest value molecules for potential cardiogenic drugs, i.e. C1.

6. Discussion and Conclusion

In the process of developing a set of experiments for discovering new cardiogenic molecules it is necessary to have a diverse set of screening models to elucidate the function, toxicity and cardiogenic effects throughout drug screening (Russell et al. 2012). Additionally it is of great value to be able to eliminate non-target molecules as quickly and as cheaply as possible (Sadek et al. 2008). Drug development is expensive, the cost of bringing a drug through clinical trials and onward to gain market approval has more than doubled according to a study from the Tufts Center for the Study of Drug Development, which pins the current cost of bringing a drug to market approval at 2.6 billion USD (Mullin et al. 2014). This high cost represent the most challenging aspect for drug development to overcome.

The goal of this thesis was chiefly to design a new set of molecule assays for drug discovery using mouse ESCs. Using an EB model is a non-specific, low cost, biological assay which can quickly show toxicity of a drug or molecule and yield introductory results for cardiogenic effects on a heterogeneous cell population. This model is an effective first stage for drug discovery. The use of DMSO (0.1%) at approximately 1% Myl2-eGFP+ as a comparative control supplies the assay with a greater than or less than measurement while simultaneously confirming that variation between individual experiments has been controlled. If an experiment does not have approximately 1% Myl2-eGFP+ cells in the DMSO control it can be repeated or thrown out because some unknown error has occurred in the protocol. Similarly the 2.5 mM treatment with Retinoic Acid acts as a negative control by knocking out CPC cells from the spontaneous differentiation. This control similarly allows the confirmation that the assay has been successful by confirming approximately 0% Myl2-eGFP+ cells in the treated control. The shortcomings of this model are that the heterogeneous cell population, while providing a good platform for toxicity, contains a few unknown elements which make it challenging to fully understand the effects of the compound. In the EB model selective toxicity may occur without affecting the total cell population, and thus go unnoticed. It can even be argued that the EB model overlooks some of the molecular mechanisms of a drug as the activity of non-mesodermal cells in the EB are not directly relevant to these assays because they do not contribute to the heart muscle. Without an endogenous reporter system such as the Myl2-eGFP reporter, utilized here, the spontaneous differentiation model would require cTnT staining for each assay. Although the heterogeneous population is more akin to an adult organism the goal

of this assay in the pipeline was to test for cardiogenic effects on cardiomyocyte differentiation and proliferation, so the effects of molecule X on bone marrow cells for example, albeit interesting, are not relevant to our intended goals. One future application of the EB model is that because the EB forms already on Day 2, unlike the directed differentiation model which can only have the first molecule addition Day 5-6, the molecules or drugs can be tested against a diverse set of early time points. Here molecule addition was on Day 2-4 and Day 6-10, as early as mesodermal induction (≅D3) and before cardiac progenitor cells form (≅D4). However, if a given molecule is thought to influence CPC genes or mesodermal induction genes a specific molecule can be tested at ≅D4 or ≅D3, respectively. This first assay offers excellent insights into exploring the unique molecular mechanism of individual molecules or gene specific molecules across developmentally relevant time points. Due to the heterogeneity of the cell population in this introductory assay it can be concluded that the EB molecule assay is most useful as a first stage assay for general toxicity in a biological model and for general cardiogenic effects, greater than or less than comparative studies. It cannot however be undoubtedly confirmed that a molecule which shows an increase in Myl2-eGFP+ cells is expressing cardiogenic effects, only an increase in the detection of the Myl2-eGFP+ cells.

For this thesis approximately 100 novel molecules from our labs archive were screened and either eliminated due to lack of cardiogenic effects or passed forward to the directed differentiation model. Throughout the experimental phase of this thesis 12 cardiogenic molecules and 12 cardiosuppressive molecules were discovered but remained unreported due to the potential for patenting and the need for further testing before their molecular mechanisms of action and chemical structure can be released. As the focus of the thesis was the design of the assays and not the molecules only an excerpt of molecules are included, specifically the molecule C1 which is reported to have cardiogenic effects in all the assays and functions as the proof of principle for the progression of a novel molecule through this novel drug discovery pipeline.

Using the directed differentiation model to screen molecules that have passed through the EB model for toxicity analysis, and show cardiogenic properties in the EB model, was an effective next step due to the increased presence of cardiomyocytes in this model. With ≅20% cardiomyocytes one can see more clearly if a molecule increases the purity or expresses cardiosuppresive effects. The drawbacks of this system is the continued presence of undefined

cell types from both the ectoderm and endoderm germ lineages in the molecular assay. These cell types are outside the spectrum of our interest and are not representative of the types of cell populations found in the adult heart. The directed differentiation assay does however provide a cardiomyocyte enriched environment and yields fully confluent beating sheets of cardiomyocytes. This layout means that the penetrance of our target drug is not an obstacle when assessing the assays viability, because diffusion will provide sufficient drug to all the cells in the monolayer. In addition the presence of beating cardiomyocytes creates a microenvironment for molecule screening which subjects the molecules to an environment more similar to the functional heart myocardium.

While the directed differentiation is more intensive to make experimentally the scalability of this assay is an advantage. After the induction of cardiac mesoderm on Day 2 - Day 4 the cells can be plated to a monolayer in any format, i.e. 96 well or 384 well plates. This allows the screening of tens or even hundreds of molecules within a single experiment. The ability to scale this assay and the potential to integrate it with: a bioreactor (for larger cell cultures), and a robotic arm (to pipette hundreds of compounds), is an attractive drug discovery model for both industry and academia. In addition the integration of a plate reader and the utilization of a reporter line allows the drug screening great automation and the prospects of reaching a high-throughput level.

Using the MACS system is effective at specifying our molecule assay for the mesoderm lineage. Using both FLK1 and PDGFRα together however is possibly too specific and does not yield enough cells to effectively screen compounds in our experiments. In future studies a larger scale operation could use this system for screening by using a much higher starting culture, ≅100 million cells and potentially utilizing a bioreactor to generate even larger numbers of pluripotent embryonic stem cells. Due to the low yield of FLK1+ and PDGFRα+ cardiac progenitor cells in this system however it is not possible to generate enough cells to have proper replicates for molecule screening. For a single experiment in a 96 well plate a minimum of 12 million cells are needed to seed the plate effectively and the most double positive cells recovered in a single experiment did not exceed 300,000 cells. Individually however the FLK1 MACS offers a molecule assay which yields enough FLK1+ cells to screen drugs but with some variation in the reproducibility of the MACS microbead system. The accuracy of FLK1 surface marker to correctly and consistently identify the FLK1+, ESC derived Day 4 CPCs, in our

model was not reproducible in the given experimental timeframe. The MACS molecule assay is dependent on correctly sorting FLK1 populations, however this data suggests that enrichment of FLK1+ multipotent cardiac progenitor cells is possible through both the depletion of FLK1+ cells or FLK1- cells. This finding could indicate that another time point for the MACS FLK1 sort, Day 4.25, could further increase the purity of cardiomyocytes and reproducibility of this assay.

The most likely improvement to this assay could be to focus more specifically on the time points of expression of the cell surface markers. Failure to identify the correct time point to sort cells as D3.25 FLK1+ marks hematopoietic progenitors and D4.25 FLK1+ marks cardiac progenitor cells (Kattman et al. 2011) could be a cause for the challenges in reproducing these MACS experiments. The narrow developmental time points, although easy to envision, are easy to miss *in vitro,* and do not necessarily aligned to *in vitro* models with different cell passages and cell lines. Further study is needed to perfect the MACS system for a MACS based cardiogenic molecule assay based on either FLK1 or PDGFRα cell surface markers. Additionally, MACS using both FLK1 and PDGFRα cell surface markers may be most advantageous to utilize in establishing a frozen stock of FLK1+/PDGFRα+ CPCs. In this system having frozen stocks would reduce the length of this assay and provide an excellent platform for expanding FLK1+/PDGFRα+ CPCs for cardiogenic molecule screening. In the course of this thesis this idea was attempted but faced challenges in the freeze/thaw process where the FLK1+/PDGFRα+ CPCs did not maintain their cellular identity upon thawing. Future studies are needed to better understand how to best freeze and thaw FLK1+/PDGFRα+ CPCs.

As a basis for a molecule assay FLK1 alone is attractive because it is a cardiac mesodermal marker on Day 4.25. Cardiac mesoderm should be present providing us with a heart-like population of cells including; smooth muscle cells, vascular endothelial cells, cardiomyocytes and cardiac fibroblast cells. This population does not specifically allow us to screen for cardiomyocyte purity but does offer an environment more similar to the cardiomyocyte microenvironment found in the adult heart. One of the most interesting newly emerging concepts from this assay model is the debate around 2D modelling in stem cell culturing. As cardiomyocytes in the adult heart never come in contact with a 2D space there is currently great debate as to the usefulness of 2D screening (Kurokawa et al. 2015) and the

potential importance of favoring 3D modeling when exploring how a drug interacts with its targeted microenvironment in the 3D space.

In establishing these assays the importance of positive and negative controls was of great interest. In order to build the best molecule assay for drug discovery, molecules must be compared to known compounds which have known pathways. In the EB model the negative control used was high concentrations of retinoic acid 2.5 µM as its primary role is the differentiation of neurons and yields ganglionic clusters *in vitro* (Janesick et al. 2015). Unfortunately the use of a positive control is impossible as there are no known single molecules known to differentiate cardiomyocytes at 100% efficiency, which would eliminate the need to design these drug discovery assays. In the directed differentiation model and the MACS model a Wnt Inhibitor and a GSK3 inhibitor were used as controls in order to manipulate the induction of cardiac mesoderm. The Wnt Inhibitor late stage (Day 7-10) exposure was the closest known molecule to a positive control used to block the Wnt pathway after Day 6 and showed an increase in cardiomyocyte purity. The GSK3 inhibitor function as a negative control which blocks GSK3, an inhibitor of canonical Wnt pathway, and allows the Wnt pathway to continue expressing outside of the targeted time point, driving the differentiation of non-myocytes. Although the lack of a true positive control presents challenges to these drug discovery assays all the molecules were dissolved in DMSO and thus DMSO offers a comparative control.

Within the context of this thesis 12 cardiogenic compounds and 12 cardiosuppressive compounds were discovered but could not be included in this thesis due to the need for patenting these compounds by the 3i Regeneration Project at the University of Helsinki. It was the goal of this thesis to present the development of the drug screening pipeline for which these drugs were discovered. This work led to the advancement of key drug screening models which have yielded both cardiogenic molecules for continued research and reaffirmed previous data on the cardiogenic effects of novel drugs on ESCs.

6.1 Future Perspectives

Future studies will likely build on this work by following a current trend in cell sphere development. There is newly emerging studies utilizing cardiospheres derived in a scaffold free environment which show promise for more accurate 3D modeling of cardiac cell types *in vitro* (Nguyen et al. 2014, Kinney et al. 2014). The development of a mesodermal cardiomyogenic

cardiosphere model would solve the shortcomings of a 2D monolayer assay while simultaneously addressing the problems with heterogeneous cell populations. A heterogeneous cell population positively selected for FLK1 and PDGFRα on a larger scale and then plated into a 3D cardiosphere model would offer both the correct mesodermal cell lineage and a complimentary microenvironment to assess the usefulness of high value drugs in the generation of cardiomyocytes. The applications of this cardiosphere model have been explored in a recent publication by Birket et al. where they focus on the patterning of cardiac progenitor cells in the cardiosphere derived from a directed differentiation model but not utilizing a MACS system (Birket et al. 2015). This group showed that cardiac progenitor cells from the directed differentiation actually mimics heart field development *in vitro,* an impressive step towards developing a clinically relevant *in vitro* model for heart development

In addition to our understanding of cardiomyocytes in *in vitro* microenvironments there is a need to develop an assay which would allow experimentation on a highly pure population. Although yielding few multipotent cardiac progenitor cells, the use of FLK1 and PDGFRα MACs could be utilized in small scale assays to explore the continued proliferation of cardiomyocytes such as in the recently published Numb and Numb-like renewal of cardiac progenitor cells. This study highlights the need to not only transplant cells to an MI patient but activate the native cardiomyocytes to leave their quiescent state and activate self-renewal in the cardiac microenvironment (Shenje et al. 2014). This group has shown that Numb protein acts as a cell signaling protein in early Day 6 expression, the same time Islet1+ expression is detected for cardiac progenitor cells. Using the FLK1 and PDGFRα MACs model would offer a unique environment to test the Numb and Numb-like proteins role in self-renewal of cardiomyocytes.

Future studies will help to further the use of cardiomyocytes in regenerative therapy for MI patients, and drug screening models will play a key role in drug discovery to enhance cardiomyocyte purity *in vitro* and the development of stem cell based therapies for damaged hearts. Alongside the *in vitro* generation of cardiomyocytes for MI patients, treatment will likely include a drug cocktail which temporally activates the required genes for cardiomyocyte proliferation, differentiation and maturation. Continued exploration of cardiogenic drugs and the molecular mechanism which drive cardiomyocyte differentiation will play a key role in

bringing drugs from discovery and development to clinical medicine, where millions of MI patients are eagerly awaiting much needed treatment.

7. Acknowledgements

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